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The U. S. P. Collaborative Cat Assays for Digitalis*

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The official assay for digitalis in the U. S. Pharmacopœia XI used frogs as the test animal; in the U. S. P. XII frogs were replaced by cats. Inability to obtain concordant results with frogs did not determine the change. As shown elsewhere (12), collaborative study in anticipation of the U. S. P. XII had developed the frog method to a stage where different collaborators could obtain quantitatively the same results from the same preparations. The frog assay failed in that a number of preparations judged as equivalent with frogs proved to be quite unequal when administered in other experiments (8) to human patients. This was more often the case with tinctures differing in age (8). Under the conditions of the assay, frog and man reacted differently to the glycosides in digitalis, so that the frog was not a reliable indicator of therapeutic potency.

If it could meet this basic requirement, the cat assay was the logical alternative. Since its introduction in 1910 by Hatcher and Brody (10), digitalis has been assayed repeatedly on cats in a large number of American and European laboratories (11, 15), so that the method is backed by long experience. As shown by the U. S. P. public hearings on the digitalis assay (14), such preparations have been preferred by many clinicians, including the New York Heart Association with its 50 member clinics. This preference has grown out of clinical experience in which cat-assayed digitalis seemed to have a more uniform potency. Recently, quantitative assays in man by Gold, Cattell and their associates (9) have confirmed these clinical impressions that preparations of digitalis standardized on cats were more nearly equipotent in man than if standardized on frogs. Further experience, however, indicates that the clinical oral potency of purified digitalis preparations cannot be predicted from cat assays alone. Since experience with quantitative human assays was too recent and facilities were too limited, animal assays could not be by-passed and a collaborative study of the cat assay was begun.

This report presents the results of three collaborative experiments on the cat assay for digitalis, covering the period from June to November, 1941, with the participation of sixteen governmental, university and industrial laboratories. All assays were made with the "New Study Powder" of the fifth comparison on frogs (12), with the addition of the International Standard Digi-

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talis (1936) in the third experiment. The "New Study Powder" later became the U. S. P. Digitalis Reference Standard (1942). Since 10 of the 16 laboratories participating in the cat assays also assayed the same preparation of digitalis or one of its precursors on frogs, a comparison of the two methods within laboratories would be possible, but has not been attempted here.

Conclusions have been based upon the data from 616 cats. As a preliminary stage the lethal dose for each cat was recomputed from the original protocols or their equivalent. Since individual cats varied in their susceptibility to digitalis, quantitative conclusions could be reached only by an extensive use of statistical method. Among these applications several are new to biological assay. Statistical analysis has had two objectives. The first was to measure the precision and accuracy of the present cat assay on the basis of its performance in these collaborative experiments. The second was to determine how the efficiency of a current assay could be increased by utilizing the information in those done previously. This has revealed some of the advantages which may be expected from such an approach.

TABLE I.—SUMMARY OF COLLABORATORS AND NUMBERS OF CATS USED FOR EACH PREPARATION IN U. S. P. CAT ASSAYS ON NEW STUDY POWDER OF DIGITALIS

Code No.		First Experiment		Second Experiment			Third Experiment	
Cat Assays	Frog Assays	Tincture	85% Tincture	Tincture	80% Tincture	Powder	Internat. Std. (1936)	Powder
1	1229	9	9	6	6	6	11	11
2	1236	9	9	6	6	6	..	..
3	..	9	9	6	6	6	9	9
4	..	9	9	12	9	6	..	..
5	..	9	9	6	6	6	..	..
6	..	8	9	7	8	6	..	..
7	1224	9	9	..	..	..	..	..
8	1227	10	9	6	6	6	12	12
9	1228	9	10	13	11	11	10	10
10[a]	1229	9	9	..	..	..	4	6
11	1222	..	..	6	6	6	6	6
13	1226	..	..	6	9	8	..	..
14	1237	..	..	9	7	6	..	..
15	1235	..	..	6	7	6	..	..
16	..	9	9	..	..	..	..	..
19	1225	..	..	10	12	10	..	..
20	..	..	..	10	8	6	..	..
Total	..	99	100	109	107	95	52	54

[a] A different operator in the same laboratory as No. 1.

Collaborators: Abbott Laboratories; Ciba Pharmaceutical Company; Cornell University Medical College; Division of Pharmacology, Food and Drug Administration; Eli Lilly and Co.; E. R. Squibb and Sons; Laboratory of Hygiene of the Canadian Department of Pensions and National Health; La Wall and Harrisson; Lederle Laboratories; Medical College of Virginia; New York University College of Medicine; Parke-Davis Co.; Syracuse University College of Medicine; Temple University Medical School; Upjohn Co.; Warner Institute for Therapeutic Research.

I. EXPERIMENTAL METHODS AND RESULTS

Preparation of Digitalis.—As has been noted, the New Study Powder was tested in all three experiments. The first experiment compared two tinctures, of which one was an undisclosed dilution of the other, to determine how closely a known relative potency could be estimated. The standard tincture was prepared by Dr. Harry Gold at Cornell University Medical College in accord with the directions adopted later in U. S. P. XII: To the weighed reference powder "add sufficient menstruum consisting of 4 parts of alcohol, by volume, and 1 part of distilled water, by volume, so that the total volume of menstruum added corresponds to 10 cc. for each Gm. of powder. . . . Shake the mixture for 24 ± 2 hours at 25° C., ±5°, by mechanical means which continuously brings the solid material into fresh contact with the liquid phase. Immediately thereafter, centrifuge the mixture and decant into a dry, hard-glass bottle having a tight closure, and preserve under refrigeration until used." For the second tincture 850 cc. of this "standard" were diluted to 1000 cc. with 80% alcohol. In Table I, which shows the number of cats run by each collaborator in each experiment, these preparations are designated as "tincture" and "85% tincture," respectively.

The second experiment tested two concentrations of a single tincture in comparison with a tincture prepared by each collaborator from the same powder by the standard technique. For this experiment, a new tincture was prepared by Professor E. Fullerton Cook in Philadelphia following the standard procedure, except that after agitation the mixture of powder and menstruum was allowed to stand for twenty-four hours and then the tincture decanted off. Part of this tincture was diluted to an 80% concentration, using 25 cc. of menstruum for each 100 cc. of tincture. Two-ounce bottles of these two liquid preparations, full-strength tincture and 80% tincture, together with 4-Gm. vials of the bulk powder, were mailed to collaborators late in July, 1941. The largest number of collaborators participated in the second experiment (Table I).

The third experiment established for cats the potency of the New Study Powder relative to International Standard Digitalis. On the basis of this

finding the New Study Powder was diluted with exhausted marc to obtain a powder of which 0.1 Gm. represents a potency equivalent to 1 International Digitalis Unit when assayed by the U. S. P. XII method. This is now the U. S. P. Digitalis Reference Standard (1942). Because of the limited time available, the samples of New Study Powder and ampuls of International Standard Digitalis (1936) were sent to a smaller group of laboratories. Collaborators were instructed to prepare tinctures for assay in accord with the directions quoted above from U. S. P. XII. By definition, 0.08 Gm. of International Standard Digitalis (1936) represents 1 International Digitalis Unit and Collaborators 1 and 11 departed from directions by preparing tinctures containing 1 International Digitalis Unit per cc. instead of the activity in 0.1 Gm. of powder. For convenience in estimating relative potencies, tinctures prepared from the International Standard Digitalis (1936) have been converted for all collaborators to terms of 1 unit per cc.

The original data in Table II a, b, c give the results for the 616 cats in the three experiments. For each individual cat the lethal dose has been expressed in cc. of tinctures per Kg. of live body weight. Hence, each cc. of tincture represents 0.1 Gm. of New Study Powder or 1 International Digitalis Unit for the cats injected with International Standard Digitalis.

The Animal Preparation.—The animals used in the collaborative assays conformed in most respects to the requirements in the U. S. P. XII. Of the 616 cats, 29 weighed less than the Pharmacopœial minimum of 2.0 Kg., but only four of these differed by more than 10 per cent from the minimum. One cat exceeded the official maximum weight of 4.0 Kg. The lethal dose for these out-size animals was consistent with that for other individuals in their respective groups, so that they have been retained in the calculations.

The U. S. P. specifies that cats are to be assigned at random to standard and unknown with the restriction that the two groups shall not differ in their average weight by more than 50 per cent. The collaborative assays fell well within this restriction. In 45 separate estimates of relative potency the difference in mean body weight averaged 8.5% between groups and only three differed by more than 25%. Differences in size have been adjusted by dividing the lethal dose per cat by its body weight, a correction which has proved satisfactory in the past (3). To test approximately whether the same relation held in the present assays, the determinations of relative potency in each laboratory and experiment have been plotted against the ratio of the mean body weight for the unknown to that for the standard tincture. No consistent trends were found which would suggest an alternative adjustment for differences in size, although a more sensitive test might have led to another result.

As was specified later in the Pharmacopœial assay, the cats were to be neither obese, emaciated, lactating, nor pregnant. Food was removed the day before use. For the assay, they were lightly anesthetized with ether, immobilized and a light anesthesia maintained throughout the injection, as judged by the presence of pupillary and corneal reflexes and tension in the voluntary musculature. The degree of anesthesia proved one of the most difficult phases of the technique to standardize and probably contributed materially to differences in the mean lethal dose between laboratories. Differences due to sex apparently were negligible but have not been tested critically in the present experiments.

Administration of the Drug.—The cat assay of digitalis is an all-or-none assay in which the lethal dose is determined separately by titration for each individual animal. A test solution of the tincture in physiological saline is injected slowly from a graduated burette, through a cannula in a femoral vein, until the cat dies from cessation of the heart beat. In most previous experiments the solution has been injected continuously. This requires frequent adjustment to maintain a uniform flow even when the changing head of solution in the burette is compensated by an attachment (4). To avoid these complications the solution was injected periodically in the collaborative assays. For a given preparation the estimated fatal dose per Kg. of cat was diluted to 15 cc. with an isotonic solution of sodium chloride on the day of the test. One cubic centimeter of the diluted material per Kg. of body weight was then injected within a few seconds and repeated at five-minute intervals until the cat died. There was no standard procedure relative to animals dying during an injection, some laboratories including the last injection, and others excluding it. Despite this discrepancy the method proved satisfactory under collaborative trial and was adopted for the pharmacopœial assay.

The rate of injection is known to modify the lethal dose of cardiac glycosides in cats (18), so that only part of the amount injected contributes to the death of the animal, especially in the case of the last injections. As the concentration of digitalis in the injection fluid is increased, the lethal dose measured experimentally tends to increase. When the injection fluid is sufficiently dilute to require on the average 15 injections at five-minute intervals, the lethal dose seems to be relatively independent of small overestimates or underestimates of its concentration.

For the first experiment, all collaborators were asked to assume a lethal dose of 0.70 cc. of tincture per Kg. for both preparations and to prepare injection fluids containing 0.70 cc. of tincture in each 15 cc. The observed mean dose exceeded 0.70 cc./Kg. by 2 to 33% in nine assay groups and fell short of this value by 1 to 26% in 11 others. Collaborator 6 misread the instructions and injected the drug at about one-half the standard rate, at 1 cc. instead of 1 cc./Kg. every five minutes. As a result the injection period in his tests averaged nearly twice as long as that of the other collaborators. Yet his mean lethal dose for the standard tincture was 80% and for the dilute tincture 89% of the estimated 0.70 cc./Kg., both within the range observed with the prescribed rate of injection. However, due to this discrepancy, the results for Collaborator 6 have been omitted from the averages for the first comparison.

The geometric mean fatal dose for full-strength tincture in the first experiment was 0.657 cc./Kg. so that in the directions for the second and third experiments the assumed lethal dose was reduced to 0.65 cc./Kg. In order to hold the injection period within narrower limits, despite variations in susceptibility, collaborators were asked to average the number of injections from the first three cats treated with each preparation. Should this average fall outside limits of 13 and 19 injections, these data were to be regarded as preliminary and test solutions at a higher or lower dilution prepared for a definitive assay. Because of these instructions, the concentration of the test solutions varied considerably, as is evident from the dilution column in Table II for the second and third experiments. In order that it may be compared directly with the doses obtained experimentally, the concentration of the injection fluid is given in terms of cc. of tincture per 15 cc. of solution. In 15 of the 59 groups in the second and third experiments two or more dilutions of the injection fluid have been used in the results reported in Table II. Only two groups averaged less than 13 or more than 19 injections; both groups were injected with a single dilution throughout and yielded esti-

TABLE IIA.—ORIGINAL DATA FROM FIRST COLLABORATIVE EXPERIMENT ON NEW STUDY POWDER

Lab. No.	Tincture Date, 1941	Wt., Kg.[a]	Sex	Fatal Dose	85% Tincture Date, 1941	Wt., Kg.	Sex	Fatal Dose	Lab. No.	Tincture Date, 1941	Wt., Kg.	Sex	Fatal Dose	85% Tincture Date, 1941	Wt., Kg.	Sex	Fatal Dose
1	6/7	2.64	..	0.513	6/7	2.98	..	0.887	7	6/12	2.34	M	0.700	6/12	2.57	M	0.653
	6/7	3.40	..	0.560	6/7	2.15	..	0.653		6/12	3.08	M	0.512	6/12	3.08	M	0.838
	6/8	2.60	..	0.747	6/8	2.53	..	0.700		6/12	2.74	M	0.701	6/12	2.51	M	0.559
	6/8	2.67	..	0.653	6/8	2.92	..	0.513		6/13	2.84	M	0.514	6/13	2.62	F	0.843
	6/8	2.60	..	0.513	6/8	2.60	..	0.840		6/13	2.67	M	0.606	6/13	2.91	M	0.653
	6/9	3.00	..	0.560	6/9	2.25	..	0.560		6/13	2.60	M	0.795	6/13	2.46	M	0.842
	6/9	2.49	..	0.373	6/9	3.30	..	0.980		6/17	2.18	M	0.654	6/17	2.17	M	0.512
	6/9	3.21	..	0.747	6/9	2.64	..	0.513		6/17	2.00	F	0.654	6/17	2.47	M	0.840
	6/9	2.11	..	0.747	6/9	2.28	..	0.653		6/17	2.46	F	0.841	6/17	2.22	M	0.793
2	6/9	2.44	F	0.845	6/9	2.62	F	0.834	8	6/13	2.60	F	0.653	6/13	2.65	F	0.674
	6/9	2.44	M	0.734	6/9	1.86	F	0.853		6/13	2.25	F	0.846	6/13	2.50	M	0.513
	6/9	1.84	F	0.771	6/9	2.54	F	0.597		6/13	1.95	F	0.700	6/13	2.40	F	0.747
	6/13	3.12	M	0.604	6/13	3.34	M	0.876		6/18	2.40	F	0.793	6/18	2.75	F	0.793
	6/13	2.52	F	0.694	6/13	3.10	F	0.700		6/18	2.40	M	0.661	6/18	2.55	M	0.793
	6/13	3.10	M	0.887	6/13	3.36	M	0.736		6/18	2.55	F	0.677	6/18	3.20	F	0.700
	6/14	3.02	F	1.020	6/14	2.20	F	0.793		6/19	3.35	M	0.700	6/19	2.40	F	0.840
	6/14	3.20	M	0.828	6/14	2.30	M	0.649		6/19	2.60	F	0.700	6/19	2.70	M	0.747
	6/14	1.70	F	0.607	6/14	2.30	F	0.795		6/19	2.00	F	0.560	6/19	3.50	F	0.560[b]
										6/20	2.90	M	0.653	6/20	3.00	M	0.840
3	6/7	3.07	M	0.684	6/7	2.74	F	0.639									
	6/7	2.26	F	0.664	6/7	2.92	M	0.771	9	6/12	2.05	F	0.669	6/11	2.30	F	0.980
	6/7	2.25	F	0.667	6/7	2.30	M	0.707		6/12	2.99	F	0.561	6/11	2.05	F	0.813
	6/13	3.10	M	0.542	6/13	2.45	M	0.621		6/13	2.98	M	0.612	6/12	3.02	F	0.832
	6/13	2.69	F	0.607	6/13	3.00	F	0.700		6/13	4.00	M	0.695	6/12	3.10	F	0.592
	6/13	2.85	F	0.618	6/13	2.16	M	0.735		6/16	2.63	M	0.617	6/13	3.69	M	0.889
	6/16	2.10	F	0.488	6/16	2.58	M	0.678		6/16	2.24	F	0.671	6/14	2.60	M	0.980
	6/16	2.99	M	0.734	6/16	2.30	M	0.659		6/16	2.52	F	0.694	6/16	3.81	M	0.559
	6/16	2.82	F	0.645	6/16	3.08	M	0.636		6/17	3.98	M	0.798	6/17	2.13	F	0.673
										6/18	2.52	F	0.788	6/17	2.74	F	0.768
4	6/10	2.75	F	0.512	6/10	2.43	F	0.791						6/18	2.04	F	1.053
	6/10	2.40	M	0.887	6/10	2.12	F	0.605									
	6/10	2.00	F	0.653	6/10	2.14	F	0.606	10	6/17	2.32	F	0.555	6/17	3.02	F	0.510
	6/11	2.59	M	0.932	6/11	2.93	F	1.061		6/17	2.44	F	0.459	6/17	2.75	F	0.412
	6/11	2.98	M	0.669	6/11	3.01	M	1.121		6/17	2.88	F	0.564	6/17	2.22	F	0.740
	6/11	3.43	M	0.653	6/11	3.01	M	0.699		6/20	2.73	F	0.646	6/20	3.32	M	0.742
	6/13	2.06	F	0.419	6/13	1.91	F	0.840		6/20	2.97	F	0.566	6/20	3.19	F	0.843
	6/13	2.07	F	0.514	6/13	2.12	F	0.746		6/20	2.52	F	0.509	6/20	2.79	F	0.609
	6/13	2.74	F	0.889	6/13	2.49	M	0.886		6/23	2.08	F	0.565	6/23	2.05	F	0.513
										6/23	2.38	M	0.471	6/23	2.08	F	0.707
5	6/12	3.0	M	0.70	6/12	3.6	F	0.98		6/23	3.48	M	0.704	6/23	2.97	F	0.566
	6/12	2.9	F	0.70	6/12	2.6	M	0.75									
	6/12	3.3	F	0.89	6/12	2.6	F	0.89	16	6/21	2.59	F	0.656	6/20	2.51	M	0.653
	6/13	3.1	M	0.70	6/13	3.2	M	1.12		6/21	2.79	F	0.793	6/20	2.44	M	0.887
	6/13	3.4	F	0.56	6/13	3.5	M	1.21		6/21	2.60	F	0.887	6/21	3.22	M	0.696
	6/13	2.8	M	0.75	6/13	3.0	F	0.89		6/21	2.88	M	0.745	6/21	2.48	M	1.027
	6/16	3.3	F	0.79	6/16	2.0	F	0.93		6/21	3.22	M	0.557	6/21	2.82	F	0.792
	6/16	2.7	M	0.84	6/16	3.1	F	0.70		6/21	3.16	F	0.653	6/21	2.05	M	0.607
	6/16	3.2	M	0.65	6/16	2.6	F	1.03		6/21	2.16	F	0.421	6/21	3.15	F	0.607
										6/21	2.47	M	0.607	6/21	2.11	F	0.653
6	6/10	1.05	..	0.622	6/10	1.8	..	0.544		6/21	2.46	M	0.699	6/21	2.54	F	0.757
		1.53	..	0.430		1.9	..	0.761									
		2.0	..	0.630		2.0	..	0.817									
		2.0	..	0.513		2.28	..	0.921									
		2.04	..	0.572		2.4	..	0.642									
		3.11	..	0.570		2.5	..	0.579									
		3.2	..	0.627		2.8	..	0.407									
	6/14	3.4	..	0.563		2.9	..	0.549									
					6/14	2.94	..	0.541									

[a] Weight is the live weight in Kg.; digitalis administered uniformly with 0.70 cc. of tincture in 15 cc. of saline injection fluid; fatal dose given as cc. of tincture per Kg. of live weight.

[b] Pregnant—animal omitted.

mates of relative potency well within the range of those observed. Since a change in injection rate within a given group did not seem to modify the lethal dose, no data have been omitted.

II. STABILITY OF THE LETHAL DOSE OF DIGITALIS IN CATS

The potency of digitalis has often been expressed in so-called "cat units." Many physicians have prescribed the drug and some manufacturers have labeled their preparations in these terms. Animal units for drugs of animal or plant origin are not uncommon in the absence of stable preparations suitable for standards. As standards were developed, however, one animal unit after another has been replaced, with a marked increase in drug uniformity. In the hands of some pharmacologists, however, the fatal dose of digitalis for cats in Mg./Kg. has seemed so stable that the "cat unit" was looked

TABLE IIb.—ORIGINAL DATA FROM SECOND COLLABORATIVE EXPERIMENT ON NEW STUDY POWDER[a]

Lab. No.	Tincture Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose	Dilute 80% Tincture Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose	Powder Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose
1	8/4	2.12	F	0.65	0.607	8/4	2.08	F	0.75	0.750	8/4	2.38	F	0.65	0.650
	8/4	2.68	F	0.65	0.697	8/4	2.43	F	0.75	1.048	8/4	2.71	M	0.65	0.607
	8/4	2.09	F	0.65	0.563	8/4	2.84	M	0.75	0.802	8/4	2.39	F	0.65	0.867
	8/7	3.98	M	0.65	0.477	8/7	2.55	F	0.75	0.650	8/7	2.75	M	0.65	0.693
	8/7	3.16	M	0.65	0.867	8/7	2.41	M	0.75	0.850	8/7	3.19	M	0.65	1.083
	8/7	2.69	F	0.65	0.650	8/7	2.68	F	0.75	0.850	8/7	3.04	M	0.65	0.693
2	8/25	2.0	F	0.65	0.639	8/25	3.06	M	0.65	0.878	8/25	2.2	F	0.65	0.520
	8/25	2.4	F	0.65	0.863	8/25	2.0	F	0.65	0.910	8/25	3.14	M	0.65	0.770
	8/25	1.86	F	0.65	0.575	8/25	2.0	M	0.65	0.737	8/25	2.0	F	0.65	0.780
	8/29	2.18	M	0.65	0.620	8/29	3.4	M	0.65	0.868	8/29	2.22	F	0.65	0.558
	8/29	2.55	F	0.65	0.556	8/29	2.32	M	0.65	1.035	8/29	2.8	F	0.65	0.774
	8/29	2.58	F	0.65	0.653	8/29	1.79	M	0.65	0.784	8/29	3.62	M	0.65	0.725
3	8/12	2.50	F	0.65	0.823	8/12	2.35	F	0.65	0.752	8/14	2.70	M	0.65	0.477
	8/12	2.76	M	0.65	0.659	8/12	2.85	F	0.65	0.617	8/14	2.61	F	0.65	0.475
	8/12	3.53	M	0.65	0.559	8/12	2.22	F	0.65	0.621	8/14	2.32	M	0.65	0.516
	8/12	3.04	M	0.65	0.770	8/12	3.13	M	0.65	0.857	8/14	3.03	F	0.65	0.644
	8/12	3.63	M	0.65	0.516	8/12	2.59	F	0.65	0.609	8/14	2.46	F	0.65	0.705
	8/12	2.75	F	0.65	0.618	8/12	2.49	F	0.65	1.001	8/14	2.60	F	0.65	0.663
4	8/8	3.8	M	0.65	0.780	8/11	3.70	M	0.65	0.867	8/13	3.53	M	0.65	0.737
	8/8	3.01	F	0.65	0.737	8/11	3.53	M	0.65	0.997	8/13	3.57	M	0.65	0.693
	8/8	2.67	F	0.65	0.953	8/11	2.68	M	0.65	0.997	8/13	3.77	M	0.65	0.737
	8/19	2.63	F	0.90	0.720	8/12	2.03	F	0.90	1.200	8/14	2.48	F	0.65	0.563
	8/19	2.56	F	0.90	0.840	8/12	3.40	F	0.90	0.780	8/14	2.38	F	0.65	0.780
	8/19	2.57	F	0.90	0.960	8/12	3.42	F	0.90	1.020	8/14	2.62	F	0.65	0.780
	8/19	2.67	M	0.85	0.793	8/12	3.02	F	0.90	0.840					
	8/19	2.42	F	0.85	0.793	8/12	2.26	F	0.90	0.960					
	8/19	2.41	M	0.85	0.850	8/12	3.02	F	0.90	0.840					
	8/20	2.30	F	0.85	1.020										
	8/20	3.05	F	0.85	0.737										
	8/20	3.01	M	0.85	0.850										
5	8/21	2.13	F	0.65	0.596	8/19	3.80	M	0.65	0.566	8/20	2.80	M	0.65	0.691
	8/21	2.50	F	0.65	0.691	8/19	2.80	F	0.65	0.870	8/20	3.35	M	0.65	0.526
	8/21	2.73	F	0.65	0.513	8/19	2.85	M	0.65	0.575	8/20	2.78	M	0.65	0.696
	8/21	3.58	M	0.65	0.578	8/19	2.80	F	0.65	0.696	8/20	3.35	F	0.65	0.570
	8/21	2.25	M	0.65	0.397	8/19	3.30	F	0.65	0.870	8/20	3.10	M	0.65	0.619
	8/21	3.83	M	0.65	0.557	8/19	3.40	M	0.65	0.783	8/20	3.80	M	0.65	0.518
6	8/19	2.2	F	0.65	0.737	8/18	3.5	M	0.65	0.634	8/18	2.9	M	0.65	0.867
	8/19	2.5	F	0.65	0.390	8/18	3.6	F	0.65	0.997	8/18	2.6	M	0.65	0.910
	8/19	3.0	F	0.65	0.693	8/18	2.5	M	0.65	1.257	8/18	3.1	F	0.65	0.867
	8/22	1.92	F	0.65	0.643	8/19	3.0	M	0.975	0.845	8/22	2.15	F	0.812	0.927
	8/22	2.0	F	0.65	0.563	8/19	3.0	F	0.975	0.780	8/22	2.0	M	0.812	0.921
	8/22	1.89	F	0.65	0.610	8/19	3.6	F	0.975	0.910	8/22	2.25	F	0.812	0.814
	8/23	3.42	M	0.65	0.607	8/23	2.64	F	0.65	0.865					
						8/23	3.32	F	0.812	0.812					
8	8/28	2.95	F	0.65	0.520	8/28	2.93	F	0.65	0.780	8/28	2.6	F	0.65	0.520
	8/28	2.0	M	0.65	0.650	8/28	2.33	F	0.65	0.693	8/28	2.4	F	0.65	0.737
	8/28	2.7	F	0.65	0.607	8/28	2.9	M	0.65	0.780	8/28	2.7	M	0.65	0.737
	8/29	3.15	M	0.65	0.693	8/29	2.50	F	0.70	0.933	8/29	2.38	F	0.65	0.563
	8/29	2.7	F	0.65	0.607	8/29	3.2	M	0.70	0.653	8/29	2.18	F	0.65	0.650
	8/29	2.3	F	0.65	0.520	8/29	2.36	F	0.70	0.793	8/29	3.29	M	0.65	0.520
9	8/6	3.7	M	0.65	0.823	8/8	2.54	F	0.81	0.813	8/6	2.41	M	0.65	0.606
	8/6	3.19	M	0.65	0.650	8/8	2.15	F	0.81	0.813	8/6	2.76	M	0.65	0.868
	8/7	1.98	M	0.65	0.477	8/8	2.85	F	0.81	1.302	8/7	2.26	M	0.65	0.781
	8/7	1.83	M	0.65	0.780	8/9	3.72	F	0.81	0.861	8/7	3.17	M	0.65	0.737
	8/9	2.74	F	0.65	0.823	8/9	2.88	F	0.81	0.867	8/8	2.46	F	0.65	0.694
	8/9	3.05	F	0.65	0.650	8/11	2.14	F	0.81	0.924	8/11	2.62	F	0.65	0.606
	8/11	2.32	F	0.65	0.520	8/11	1.94	F	0.81	0.649	8/11	1.98	F	0.65	0.691
	8/11	2.49	F	0.65	0.607	8/12	3.25	M	0.81	0.867	8/11	2.77	F	0.65	0.737
	8/12	2.52	F	0.65	0.909	8/12	3.13	M	0.81	0.760	8/11	1.98	F	0.65	0.779
	8/12	1.99	F	0.65	0.563	8/12	3.48	M	0.81	0.921	8/12	2.80	F	0.65	0.520
	8/12	3.41	M	0.65	0.867	8/13	2.69	M	0.81	0.705	8/13	2.22	F	0.65	0.694
	8/13	2.37	F	0.65	0.823										
	8/13	4.14	M	0.65	0.737										

TABLE IIB.—ORIGINAL DATA FROM SECOND COLLABORATIVE EXPERIMENT ON NEW STUDY POWDER

(*Continued*)

Lab. No.	Tincture					Dilute 80% Tincture					Powder				
	Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose	Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose	Date, 1941	Wt., Kg.	Sex	Dil., Cc.	Fatal Dose
11	9/3	2.55	M	0.60	0.800	9/3	2.35	M	0.90	1.026	9/3	1.98	F	0.75	1.010
	9/20	1.81	..	0.80	0.705	9/3	1.96	M	0.90	0.735	9/3	2.47	M	1.00	0.813
	9/20	2.55	F	0.80	0.725	9/5	2.27	M	0.92	0.932	9/20	2.01	F	0.80	0.742
	9/20	2.27	F	0.80	0.703	9/5	4.03	F	0.92	0.973	9/20	3.31	F	0.80	0.638
	9/22	2.32	F	0.75	0.791	9/5	2.10	M	0.92	1.100	9/20	1.81	F	0.80	0.742
	9/22	1.81	F	0.75	0.796	9/22	2.21	F	0.92	0.793	9/20	3.01	M	0.80	0.744
13	8/11	3.11	F	0.65	0.648	8/11	2.95	F	0.65	0.650	8/12	3.61	M	0.65	0.497
	8/11	2.90	F	0.65	0.520	8/12	3.66	M	0.65	0.824	8/12	3.13	M	0.65	0.520
	8/12	4.00	M	0.65	0.607	8/12	2.92	M	0.65	1.033	8/13	3.45	M	0.55	0.587
	8/14	2.12	F	0.65	0.433	8/13	3.23	M	0.75	0.793	8/13	2.99	F	0.55	0.368
	8/14	2.35	M	0.65	0.780	8/13	2.81	M	0.75	0.750	8/13	2.67	M	0.55	0.494
	8/18	2.77	F	0.65	0.569	8/13	2.80	F	0.75	0.502	8/14	2.39	F	0.55	0.589
						8/14	2.68	F	0.75	0.806	8/14	2.12	F	0.55	0.571
						8/14	2.12	F	0.75	0.941	8/18	3.11	F	0.55	0.551
						8/18	3.74	M	0.75	0.929					
14	8/8	2.25	F	0.65	0.520	8/18	3.20	F	0.65	0.737	8/28	3.23	M	0.65	0.637
	8/11	2.55	F	0.65	0.520	8/18	2.61	F	0.65	0.693	8/28	2.14	F	0.65	0.477
	8/11	3.77	M	0.65	0.520	8/18	2.3	F	0.65	0.563	8/28	2.19	F	0.65	0.477
	8/12	2.26	M	0.58	0.657	8/18	2.52	F	0.65	0.520	8/29	2.57	F	0.65	0.520
	8/12	2.4	M	0.58	0.580	8/26	2.88	F	0.65	0.650	8/29	2.67	F	0.65	0.693
	8/12	2.37	F	0.58	0.503	8/27	2.65	M	0.65	0.607	8/29	3.56	F	0.65	0.607
	8/12	2.63	F	0.58	0.503	8/27	3.02	M	0.65	0.737					
	8/29	2.89	F	0.58	0.619										
	9/3	2.46	F	0.58	0.503										
15	9/4	2.6	F	0.65	0.696	9/4	4.2	M	0.65	0.913	9/4	2.2	M	0.65	0.630
	9/4	2.6	F	0.65	0.609	9/4	2.4	F	0.65	1.033	9/4	3.2	M	0.65	0.674
	9/4	2.1	F	0.65	0.522	9/4	3.9	M	0.65	0.819	9/5	2.0	M	0.65	0.739
	9/4	2.8	F	0.65	0.562	9/5	3.7	M	0.83	1.000	9/5	2.3	M	0.65	0.696
	9/4	2.2	F	0.65	0.826	9/5	3.5	F	0.83	0.833	9/5	2.7	F	0.65	0.609
	9/5	3.8	F	0.65	0.522	9/5	2.5	M	0.83	0.833	9/5	2.2	F	0.65	0.783
						9/5	3.4	M	0.83	0.778					
19	8/18	2.96	M	0.675	0.730	8/19	3.04	F	0.675	1.021	8/18	2.0	M	0.675	0.630
	8/18	2.18	F	0.675	0.904	8/19	2.15	F	0.675	1.013	8/18	3.13	F	0.675	0.763
	8/19	2.20	F	0.675	0.720	8/19	3.70	M	1.05	1.050	8/18	2.03	F	0.675	0.621
	8/19	2.63	F	0.675	0.647	8/19	3.30		1.05	0.910	8/18	2.85	M	0.675	0.707
	8/19	2.60	F	0.675	0.807	8/19	2.92	M	1.05	1.112	8/19	3.00	F	0.675	0.675
	8/20	3.45	F	0.675	0.931	8/20	2.75	F	1.05	1.306	8/20	3.50	M	0.675	0.720
	8/26	1.94	F	0.675	0.705	8/20	2.55	M[b]	1.05	0.755	8/26	2.55	M	0.675	0.682
	8/26	3.62	M[b]	0.675	0.671	8/20	3.03	F	1.05	0.901	8/26	3.3	M[b]	0.675	0.630
	8/26	2.44	F	0.675	0.797	8/26	2.57	M	1.05	0.850	8/26	2.40	F	0.675	0.675
	8/26	2.48	M	0.675	0.680	8/26	3.40	M	1.05	1.260	8/27	2.60	M	0.675	0.765
						8/26	2.27	F	1.05	1.064					
						8/27	2.55	M	1.05	1.167					
20	8/18	3.87	M	0.57	0.460	8/14	2.15	M	0.65	0.710	8/13	2.60	F	0.65	0.606
	8/18	2.72	F	0.57	0.453	8/14	3.08	M	0.65	0.698	8/13	2.53	M	0.65	0.642
	8/18	2.67	M	0.57	0.615	8/14	1.87	F	0.65	0.705	8/13	2.99	M	0.65	0.609
	8/18	3.10	F	0.57	0.570	8/14	2.82	F	0.65	0.775	8/13	2.46	M	0.65	0.528
	8/18	2.50	F	0.57	0.494	8/14	2.52	F	0.65	0.946	8/13	2.11	F	0.65	0.689
	8/18	3.32	M	0.57	0.378	8/14	2.75	M	0.65	0.485	8/13	2.19	M	0.65	0.697
	8/18	2.22	M	0.57	0.527	8/20	2.38	F	0.65	0.655					
	8/18	2.60	F	0.57	0.722	8/20	2.85	F	0.65	0.838					
	8/20	2.97	F	0.57	0.576										
	8/20	3.10	F	0.57	0.563										

[a] Data uniform with those in Table IIA, except that dilution in cc. of tincture per 15 cc. of injection fluid is shown for each cat. With few exceptions the dilution has been smoothed to 2 significant figures, but where the original protocols gave the dilution to a third decimal, the more precise values have been used in computing the fatal dose.

[b] Castrates.

upon as the exception to the rule. Although the collaborative cat assays for digitalis were comparative experiments for testing a technique based upon a reference standard, they provide information as to the stability of the lethal dose of digitalis in cats. In frogs the median systolic dose of digitalis has proved so variable that the reference standard has had to be tested in parallel with every sample or "unknown," just the reverse of the constancy implied in the "cat unit." Before defining an assay technique we need to know where cats belong in this range. If less variable than frogs, just how stable is their susceptibility to digitalis?

TABLE IIc.—ORIGINAL DATA FROM THIRD COLLABORATIVE EXPERIMENT ON NEW STUDY POWDER

Lab. No.	International Standard[a] Date, 1941	Weight, Kg.	Sex	Dil., Units	Fatal Dose	New Study Powder Date, 1941	Weight, Kg.	Sex	Dil., Cc.	Fatal Dose
1	9/29	2.46	M	0.60	0.671	9/29	2.40	F	0.75	0.900
	9/29	2.24	F	0.60	0.760	9/29	2.85	F	0.75	0.650
	9/29	2.93	F	0.60	0.720	9/29	2.35	F	0.75	0.600
	10/3	3.81	M	0.60	0.840	10/3	2.86	F	0.75	0.600
	10/3	2.85	M	0.60	0.760	10/3	2.91	F	0.75	0.700
	10/3	2.07	F	0.60	0.520	10/3	2.78	F	0.75	0.700
	11/5	3.25	M	0.75	0.800	11/5	2.27	F	0.65	0.823
	11/5	3.53	M	0.75	0.950	11/5	3.01	M	0.65	0.693
	11/5	2.04	F	0.75	0.700	11/5	1.96	F	0.65	0.563
	11/5	2.12	F	0.75	0.950	11/5	3.36	M	0.65	0.563
	11/5	2.68	F	0.75	0.700	11/5	2.34	F	0.65	0.693
3	9/30	3.79	M[b]	0.938	0.792	9/30	2.45	M	0.65	0.619
	9/30	3.60	M	0.938	0.790	9/30	3.08	F	0.65	0.591
	9/30	2.16	F	0.938	0.868	9/30	3.92	M	0.65	0.752
	9/30	3.40	F	0.938	0.836	9/30	2.90	M	0.65	0.583
	9/30	3.42	M	0.938	0.768	10/3	2.90	F	0.65	0.583
	10/3	3.00	M	0.812	0.758	10/3	2.11	M	0.65	0.616
	10/3	2.20	F	0.812	0.936	10/3	2.30	F	0.65	0.801
	10/3	3.18	F	0.812	0.767	10/3	3.73	M	0.65	0.831
	10/3	2.88	F	0.812	1.018	10/3	3.13	F	0.65	0.623
8	10/1	2.5	M	0.688	0.687	10/1	2.8	F	0.65	0.693
	10/1	2.4	F	0.688	0.871	10/1	2.45	F	0.65	0.563
	10/1	2.0	M	0.688	0.596	10/1	2.1	F	0.65	0.780
	10/3	2.8	M	0.688	0.962	10/1	2.35	F	0.65	0.563
	10/3	2.25	F	0.688	0.733	10/1	2.7	M	0.65	0.650
	10/4	2.0	F	0.688	0.779	10/1	2.8	F	0.65	0.650
	10/13	2.1	F	0.75	0.750	10/13	2.1	F	0.65	0.520
	10/13	2.3	M	0.75	0.750	10/13	3.5	F	0.65	0.650
	10/13	2.65	F	0.75	0.750	10/13	3.1	M	0.65	0.607
	10/14	2.8	M	0.75	0.650	10/14	2.0	F	0.65	0.650
	10/14	2.1	F	0.75	0.750	10/14	2.5	F	0.65	0.607
	10/14	2.4	M	0.75	0.650	10/14	2.05	F	0.65	0.780
9	10/9	3.78	M	0.712	0.904	10/9	3.78	M	0.65	0.738
	10/9	3.16	M	0.712	0.854	10/9	3.21	M	0.65	0.693
	10/9	3.48	M	0.712	0.714	10/17	2.43	M	0.65	0.737
	10/10	2.74	M	0.712	0.857	10/17	1.99	M	0.65	0.608
	10/10	3.68	M	0.712	0.760	10/17	2.26	M	0.65	0.563
	10/10	3.55	M	0.712	0.722	10/17	3.11	M	0.65	0.563
	10/10	3.32	M	0.712	0.945	10/18	1.87	M	0.65	0.608
	10/10	3.23	M	0.712	0.760	10/18	2.03	M	0.65	0.737
	10/10	2.91	M	0.712	0.758	10/18	2.73	M	0.65	0.692
	10/11	3.52	M	0.712	0.850	10/18	2.02	M	0.65	0.651
10	7/29	2.12	F	0.625	0.867	7/28	2.91	F	0.65	0.563
	7/29	2.35	M	0.625	0.733	7/28	3.24	F	0.65	0.472
	7/30	2.15	F	0.625	0.775	7/29	3.56	M	0.65	0.615
	7/30	2.19	M	0.625	0.629	7/29	3.80	M	0.65	0.641
						7/29	2.17	F	0.65	0.485
						7/30	2.11	M	0.65	0.646
11	10/9	3.18	F	0.75	0.549	10/9	2.72	M	0.75	0.550
	10/9	3.34	M	0.75	0.850	10/9	3.34	M	0.75	0.704
	10/9	2.77	F	0.75	0.708	10/9	2.04	M	0.75	0.703
	10/10	2.55		0.75	0.867	10/10	2.04	F	0.75	0.735
	10/12	3.15	F	0.75	0.914	10/10	2.89	M	0.75	0.652
	10/12	3.00	F	0.75	0.900	10/10	2.55	M	0.75	0.561

[a] Data uniform with those in Table IIb, except that for the International Standard Digitalis, both the dilution and the fatal dose are given in International Digitalis Units rather than in cc. of standard tincture. As before, the New Study Powder was made into standard tincture at the rate of 0.1 Gm. per cc.

[b] Castrates.

The Unit for Statistical Analysis.—The logarithm of the individual lethal dose proved to be the most satisfactory unit for examining the data in Table II in respect to both the stability of the response and the relative potency of the preparations. These questions deal primarily with proportionate rather than absolute changes in response. The difference in the fatal dose of drug in two laboratories, for example, has little meaning unless related to the quantities which are being compared. Since interest is focused on relative magnitudes, logarithms enable us to turn proportions and ratios into differences, which are easier to handle statistically. Then the antilogarithm of the difference between the mean log-doses for two similar tinctures is their relative potency. In the present case the task of

looking up the logarithm of each lethal dose was more than repaid before the calculations were completed.

A more important reason for the logarithmic scale relates to statistical theory. The individual lethal doses measured experimentally form a sample drawn from a "population" of similar doses. The population is the collection of lethal doses which would result if the experiment could be continued under identical conditions with an indefinitely large number of cats. Since the number cannot be increased indefinitely, the population of doses can only be known through the sample.

The questions involved in the collaborative cat assays fell in several groups. Some were solved by determining whether two or more samples could be considered as if drawn from the same population, the so-called "tests of significance." Tests for the stability of the lethal dose, for example, belonged in this category. Other questions were answered by characterizing the population from which a sample had been drawn, such as one of relative potencies. This is known as "estimation" and includes not only the calculation of statistics which describe the population, but also of zones showing the confidence they merit as estimates of the unknown population values.

Biological data can be analyzed more easily if they conform to one of the basic patterns or "distributions" arising by random sampling. When this occurs, a long series of records may be summarized fully by very few statistics. In the present case, the "normal curve of error" is the appropriate random distribution. The logarithm of the individual fatal dose has a second advantage as the unit for statistical analysis in that it is distributed normally. In consequence the mean log-dose is also the logarithm of the median lethal dose (the log-LD_{50}), the standard deviation is independent of the mean and the pertinent information in any series of observations can be summarized by the mean, the standard deviation (or its square, the variance) and the number of observations.

Evidence for the normality of the log-dose comes from several sources. By analogy with the many dosage-effect curves for other all-or-none reactions, the log-dose rather than the dose would be expected to follow the normal distribution. A typical case, for example, is the overnight frog assay for ouabain (2). Moreover, the same relation has been shown previously in the titration technique for cardiac glycosides in cats. Graphic evidence of the skewness or asymmetry of the dose and the symmetry or normality of the log-dose has been given for ouabain in cats (2). In a longer series of 208 individuals injected with digitalis where the animals were tested in groups of four, group means differed more from one another than would be expected from the variation within groups. To bring all observations to a common base, the difference between the mean for each group and the general mean was reduced to that expected from the variation within groups. The distribution of the log-doses then proved consistent with the normal curve by both numerical and graphic test (3).

This point was examined again with the data for the two tinctures from the second experiment in Table II. From its known concentration the factor −0.0969 (the logarithm of 0.8) was added to the log-dose for each of the 107 cats tested with the dilute tincture to place them on the same basis as the 109 cats injected with full-strength tincture.

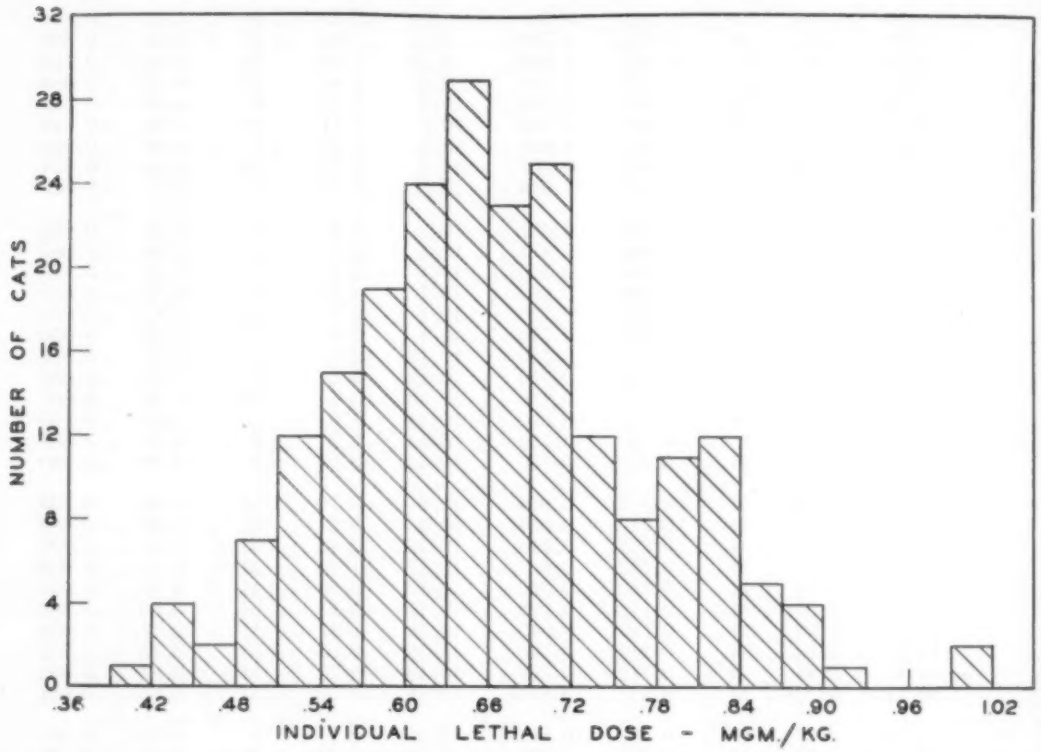

Fig. 1.—Frequency distribution of the individual lethal doses obtained with a single tincture of digitalis in 216 cats of the second experiment, adjusted for differences between laboratories.

As will be shown presently, the variance between the 14 laboratories participating in this comparison exceeded by tenfold that within laboratories, which necessitated a further adjustment. A second factor added to all log-doses reported by each laboratory reduced the difference between its mean and the general mean to 0.316 of the original value. The factor 0.316 was the ratio of the standard deviation within laboratories and tinctures to that between laboratories or 0.0720/0.2282 = 0.316. With this adjustment, the data for all 216 cats could be considered as a single sample.

Two frequency distributions were then prepared. In the first (Fig. 1) the data were scored into 21 classes on an equal arithmetic scale of doses, differing from one another by 0.03 mg./Kg. It is evident by inspection that the distribution is skewed toward one side. The numerical test for symmetry (6) shows that the skewness was statistically significant ($g_1 = 0.336 \pm 0.166$). In the second diagram (Fig. 2), the same data have been scored into 22 classes on an equal logarithmic scale of doses differing by 0.02 log-dose. Here the asymmetry has disappeared and the observations agree with the smooth curve for the normal distribution, neither statistical test for non-normality exceeding its standard error ($g_1 = -0.145 \pm 0.166$ and $g_2 = 0.174 \pm 0.330$).

An important characteristic of the normal distribution is the independence of the mean and standard deviation. Whether the standard deviation in fact was here independent of the mean has been tested by covariance with the data from the first experiment. Omitting Collaborator 6, nine or ten cats were treated with both tinctures in each laboratory, so that all 20 groups were of nearly equal validity. As computed from the log-doses for each group, the standard deviations varied from 0.0313 to 0.1218 (Table VII), and the means from 0.744 to 0.969. The mean log-dose was found to have no effect whatever upon the standard deviation, as would be expected if the log-dose of digitalis were distributed normally.

Thus the present data confirm past experience that with the logarithm of the individual lethal dose as the unit, the distribution is normal and the stability of the mean or log-LD_{50} can be considered independently of that of the standard deviation. In Section II we are concerned primarily with the variability of group means and in Section III with that of standard deviations.

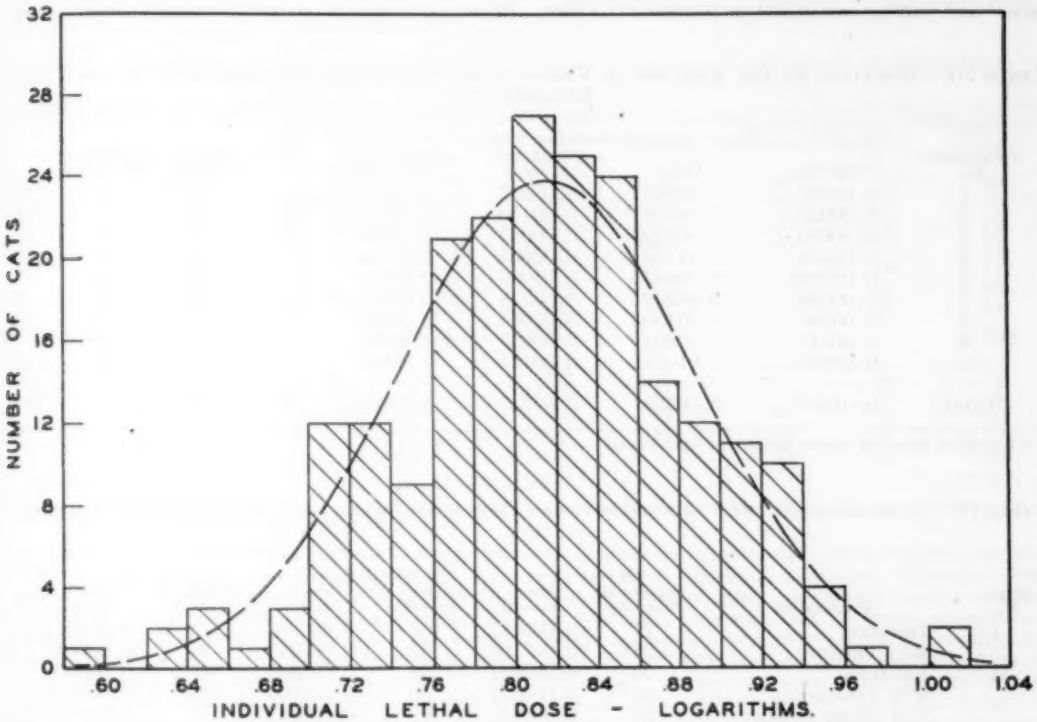

Fig. 2.—Frequency distribution on an equal logarithmic scale of the data shown in Fig. 1. The smooth curve is that expected from the theoretical normal distribution.

Variability between Days within Groups.—The first problem concerned the variation in the log-dose for a given preparation over relatively short periods of time. The first experiment was so planned that the effect of differences between days within laboratories could be segregated. Three cats were to be treated with standard tincture and three with the diluted tincture on each of three days. Several collaborators did not adhere to these instructions. Among those injected with dilute tincture by Collaborator 8 one cat proved to be pregnant and was omitted. To equalize the numbers on both preparations, the cat of most nearly the same weight was dropped from the parallel group treated with full-strength tincture. The single cat reported by Collaborator 9 on June 14 was omitted in analyzing his unbalanced tests for the effect of days by the statistical method known as "fitting constants" (19). Since 16 of 18 cats were tested by Collaborator 16 on a single day, his data were not suitable for an analysis of day-to-day variation and

have been omitted from the computation, together with those of Collaborator 6.

With the above omissions, the data from each of nine collaborators have been analyzed separately and the results summarized in Table III. In most cases three cats were treated with each tincture on the same day, giving six subgroups each of three cats. Since the three animals comprising a subgroup differed from one another primarily in their inherent susceptibility to digitalis, the variation within subgroups served as the experimental error for each collaborator. These values are listed in the last column of mean squares.

The remaining variation was that between the subgroups. This has been split into three parts in Table III. The first of these, the effect of concentration of tincture, is given in the column for "tinctures" and will be considered in Section IV. The twenty-six days between the first and last cats reported by the experimenter. Since none of these three series was appreciably more variable between days than within days, they have been included on the same basis as the assays completed within a ten-day period.

Without parallel tests, overall differences in susceptibility could not be separated readily from the interaction of days by preparations. Hence the variation in the log-doses for each preparation was divided into two parts, that between days and that within days. No collaborator showed a significant or near-significant discrepancy between days in separate analyses of variance, so that the sums of squares for all collaborators have been totaled to obtain the mean squares shown in Table IV. The variability between days was clearly negligible, and in two instances enough smaller than

TABLE III.—ISOLATION BY THE ANALYSIS OF VARIANCE OF DIFFERENCES BETWEEN DAYS IN THE FIRST EXPERIMENT

Collaborator No.	Mean Square for Variation Due to				Degrees of Freedom for Each Mean Square			
	Tinctures	Days	Tinctures × Days	Error within Subgroups				
1	0.01895	0.00083	0.00515	0.01283	1	2	2	12
2	0.00025	0.00046	0.00150	0.00562	1	2	2	12
3	0.00665	0.00242	0.00085	0.00209	1	2	2	12
4	0.03234	0.01710	0.00968	0.01149	1	2	2	12
5	0.05336[a]	0.00047	0.01026	0.00454	1	2	2	12
7	0.00598	0.00202	0.00378	0.00751	1	2	2	12
8	0.00296	0.00130	0.00664	0.00326	1	3	3	10
9	0.00447	0.00895	0.00605	0.00309	1	5	4	7
10	0.00824	0.01051	0.00417	0.00657	1	2	2	12
Total	0.01480[a]	0.00529	0.00547	0.00655	9	22	21	101

[a] Exceeded the error within subgroups significantly.

TABLE IV.—COMPARISON OF THE VARIANCE BETWEEN DAYS WITH THAT WITHIN DAYS FOR EACH PREPARATION

Expt. No.	Preparation	Between Days		Within Days		Total	
		Degrees of Freedom	Mean Square	Degrees of Freedom	Mean Square	Degrees of Freedom	Mean Square
I	Tincture	21	0.003172	60	0.006951	81	0.005971
	85% Tincture	24	0.007198	57	0.006415	81	0.006647
II	Tincture	26	0.002831	69	0.005900	95	0.005060
	80% Tincture	23	0.003651	70	0.005905	93	0.005347
	New Study Powder	20	0.003254	61	0.003947	81	0.003776
III	International Standard	10	0.003407	34	0.003549	44	0.003517
	New Study Powder	8	0.002723	38	0.003504	46	0.003368

second source of variation was the overall change in susceptibility from one day to another. As shown in the column for "days," the variance between days never exceeded that within days or subgroups significantly, and its pooled value for all collaborators was smaller than the corresponding value for error. The third source of variation between subgroups was the variability from one day to another in the estimate of relative potency, or the interaction of tinctures by days. This mean square also averaged less than the error. From the analysis of variance in Table III it could be concluded that over a period of seven to nine days or less the susceptibility to digitalis did not change enough to require simultaneous tests of standard and unknown.

Because the daily change in susceptibility proved negligible in the first study, collaborators were requested merely to complete later experiments within a ten-day period. The observations of three collaborators in the second experiment (Nos. 4, 11 and 14) exceeded these limits up to a spread of

the variation between cats tested on the same day to suggest that the variability may have been increased by some factor or factors operating through the day. In further calculations differences between days within comparisons have been ignored, except that Collaborators 1 and 8 have each been credited with two separate assays in the third experiment.

Variability Between Successive Assays.—Over periods of ten days or less, the cats used in the collaborative assays did not change in their susceptibility to digitalis. Can this stability be relied upon for longer periods? In another series (3) covering eighteen months, cats injected with digitalis at the same time of the same day were found to react more nearly alike than those treated on different days. Since the New Study Powder was used throughout the collaborative assays, the stability of the mean log-dose could be examined over the longer interval between experiments. The median lethal doses (LD_{50}'s) have been com-

pared with respect to (*a*) tinctures in the first and the second experiments, and (*b*) powder in the second and third experiments.

(*a*) Seven laboratories reported data from prepared tinctures in both the first and second experiments. For the first experiment, the mean log-dose in each laboratory was computed from only the nine or ten cats injected with test solutions prepared from full-strength tincture. For the second experiment, the individual log-doses obtained with the 80% tincture were adjusted by adding to each the logarithm of 0.8 (−0.0969) before computing the mean log-dose of each laboratory based upon 12 to 24 tincture-tested cats. The difference between each pair of mean log-doses is given in the upper part of Table V, together with the antilogarithm of the difference or the relative susceptibility (last column).

change in susceptibility was statistically significant, that for Collaborator 11 with nineteen days between tests. In general the reaction to digitalis changed less in the interval from the second to the third experiment than in that from the first to the second.

It is clear from Table V that the stability in the lethal dose of digitalis which had been observed over a period of ten to fifteen days did not extend indefinitely. This would justify the time limit in the present U. S. P. monograph. In some laboratories, however, the lethal dose may vary no more over long periods than would be expected from cats run simultaneously. Where this can be demonstrated statistically and confirmed by periodic test, estimates of potency will be more reliable if the mean log-dose for the reference standard is based upon the accumulated experience of the laboratory rather than upon the mean of a single group of six or eight cats.

TABLE V.—CHANGE IN THE MEAN LOG-DOSE OF DIGITALIS IN THE SAME LABORATORY FROM THE FIRST TO THE SECOND AND FROM THE SECOND TO THE THIRD EXPERIMENT

Change in $\bar{y}$	Lab. No.	Difference in Means	Days between Tests	Test for Significance of the Difference t	n	Relative Susceptibility
From first to second experiment	1	0.0388 ± 0.0393	56	0.99	18	1.09
	2	−0.0603 ± 0.0298	72	2.03	18	0.87
	3	−0.0059 ± 0.0320	57	0.18	18	0.99
	4	0.0822 ± 0.0374	56	2.20[a]	27	1.21
	5	−0.1119 ± 0.0324	64	3.46[a]	18	0.77
	8	−0.0574 ± 0.0216	69	2.65[a]	19	0.88
	9	0.0089 ± 0.0302	49	0.30	30	1.02
Average		−0.0274 ± 0.0250	..	...	..	0.939
From second to third experiment	1	−0.0397 ± 0.0483	53	0.82	10	0.91
	3	0.0620 ± 0.0359	47	1.73	13	1.15
	8	0.0218 ± 0.0363	33	0.60	10	1.05
	9	−0.0250 ± 0.0242	57	1.03	19	0.94
	11	−0.0778 ± 0.0348	19	2.24[a]	10	0.84
Average		−0.0132 ± 0.0277	..	...	..	0.970

[a] Difference significantly larger than its error.

If the susceptibility of cats to digitalis were stable over the interval of forty-nine to seventy-two days from the first to the second experiment, the differences in the means should not differ significantly from zero. In four laboratories the mean log-dose increased and in three laboratories it decreased. From the weighted average of the differences, the susceptibility to digitalis in the second experiment apparently agreed with that in the first within the experimental error. However, by dividing each difference by its error to obtain the statistic t in the fifth column of Table V, we have a critical test of the difference for each laboratory. In three of the seven laboratories the change in susceptibility was statistically significant, and in one other it approached significance. It is evident that even in a given laboratory the susceptibility of cats to the same preparation of digitalis may increase or decrease significantly within a period of eight weeks.

(*b*) A similar comparison was made from the results of five collaborators on New Study Powder in the second and third experiments, although discrepancies in this case could be due to differences in preparing the tincture rather than to changes in animal suceptibility. Here the interval between the last cat tested in the second experiment and the first cat in the third experiment varied from nineteen to fifty-seven days. For Collaborators 1 and 8 only the first six cats tested on the New Study Powder in the third experiment have been used for this comparison. As shown in the lower part of Table V, the positive and negative changes in susceptibility largely balanced one another. One

Variability between Collaborators.—Granted a constant method of injection, standardization in terms of "cat units" assumes that the mean susceptibility of the test animal for a given sample of drug does not vary either with time or with the laboratory or operator. As shown above, the mean lethal dose proved sufficiently stable to justify a spread of fifteen days in the conduct of an assay, but not a blanket limit for periods much longer than this. What about the other assumption in the "cat unit?" Did the lethal dose vary between collaborators when all used the same technique? If the mean log-dose varied from one collaborator to another, did they agree as to the relative potency of two preparations tested under similar conditions? These questions have been answered by the analysis of variance.

In Table VI the variation in the lethal dose has been separated into four parts for each of the three experiments. It facilitated the calculation to have equal numbers of cats on both preparations for each collaborator, so that two cats, as previously noted, have been omitted from the records of Collaborators 8 and 9 in the first experiment and all of the data for Collaborator 10 in the third experiment. In the second experiment the data on powder have been omitted and the dilution of tincture adjusted to a full-strength basis in computing the mean square for differences between collaborators.

The first row of Table VI measures the variation in the lethal dose between collaborators, independently of differences between preparations. Among the ten collaborators of the first experiment and the

14 collaborators of the second experiment the cats varied in their average susceptibility far more than would be expected from the error. The five collaborators in the third assay, on the other hand, differed no more from one another than would be expected from the variation within laboratories. Even though several observers may report the same lethal dose for a given preparation of digitalis, different laboratories cannot be relied upon to agree in this respect, especially as more laboratories are involved. The assumption in the "cat unit" of a stable lethal dose of digitalis is not justified.

For the determination of *relative* potency, however, the cat method performed very well in all three experiments. The combined results established a highly significant difference in potency in the first comparison between a tincture and an 85% concentration of the same tincture, in the second comparison between a second tincture and its dilution to 80%, and in the third comparison between International Standard Digitalis and New Study Powder. These differences are demonstrated by the large variance ratios (F) in the second row of Table VI. Despite unequal levels of susceptibility for digitalis, all collaborators assigned the same relative potency to each pair of preparations, well within the limits of the experimental error. The proof of this is in the third row of Table VI, where the mean square for the interaction of collaborator by preparation is less than that for error in all three experiments. When unknown tinctures were compared with a reference standard, collaborators agreed very well in their estimates of relative potency, even though they differed as to the lethal dose or "cat unit" for a given preparation.

The Median Lethal Dose of Digitalis Reference Standard in Cats.—Although the median lethal dose (LD_{50}) of digitalis is too variable to permit the standardization of official digitalis preparations in "cat units," its value must be known approximately in order to run a comparative assay. The U. S. P. XII requires that the estimated fatal dose per Kg. of cat be diluted to 15 cc. with isotonic solution of sodium chloride. Then one-fifteenth of the diluted material for each Kg. of body weight is to be injected every five minutes until the cat dies. The LD_{50} observed in the collaborative assays provides a preliminary estimate of the average lethal dose for future assays.

As computed from 540 cats in all three experiments, the mean log-dose or log-LD_{50} for all series on the New Study Powder was -0.1886. When corrected for the factor used in preparing the U. S. P. Digitalis Reference Standard and converted from logarithms to cc. of standard tincture, this gives the geometric mean or LD_{50} in cats as 0.77 U. S. P. Digitalis Unit. Until its value is known for a given laboratory and season, this may be used as a provisional estimate of the fatal dose per Kg. Its approximate standard error has been computed from the average variances between and within collaborators in Table VI, which gives for the LD_{50} of six-cat groups a standard error of ± 0.09 Digitalis Unit.

III. CONSISTENCY OF THE VARIATION IN SUSCEPTIBILITY

In the preceding section we have been concerned with the instability of the mean log-dose of digitalis in cats. This is only part of the story. Differences between means were interpreted by comparison with the variation between individuals. In some cases, as in Tables III and V, the variation or "error" has been computed separately for each laboratory; in others, as in Tables IV and VI, the variation among the individual cats in several laboratories has been combined. The present section considers the constancy in the variation of the individual log-doses about group means. Because the log-doses agree with the normal distribution, this variation is measured in terms of the standard deviation s or of its square, the variance or mean square for error. For a given group of N cats, the variance is computed by the equation

$$s^2 = \frac{S(y - \bar{y})^2}{N - 1} = \frac{S(y^2) - \bar{y}S(y)}{N - 1} = \frac{[y^2]}{N - 1} \quad (1)$$

where y is the individual log-dose obtained with a given preparation of either reference standard or unknown, $\bar{y}$ is the mean of the y's, computed to six or more decimal places, and S the sign for summation. The numerator, $[y^2]$, is known as the "sum of squares." The standard deviation s is equivalent to the reciprocal of the slope of the dosage-effect curve ($s = 1/b$) in the frog assay for digitalis, when the dose is plotted in terms of logarithms and the effect in probits. The magnitude of the standard deviation determines how many individuals are needed to assay any given sample of drug with a specified precision.

The mean log-dose as computed from a given group of cats is an estimate of the log-LD_{50}. In determining potency as the antilogarithm of the difference between two such means, it is assumed that their relative values are constant whether compared at LD_5 or at LD_{95}. This is true, however, only if they have the same standard deviation or variance. Since in the normal distribution the standard deviation is independent of the mean, factors known to modify one affect the other independently if at all. Of the two the standard deviation is usually the more stable when compared with its expected variation. The first problem is to determine how far this stability extended in the cat assays for digitalis.

TABLE VI.—ANALYSIS OF VARIANCE IN THE LETHAL DOSE OF DIGITALIS BETWEEN THE TINCTURE OF THE FIRST AND SECOND EXPERIMENTS AND THE POWDERS OF THE THIRD EXPERIMENT, WITH OMISSIONS NOTED IN THE TEXT

	First Experiment			Second Experiment			Third Experiment		
Variation	Degrees of Freedom	Mean Square	F	Degrees of Freedom	Mean Square	F	Degrees of Freedom	Mean Square	F
Between collaborators	9	0.03162	4.99	13	0.05209	10.01	4	0.00339	0.99
Between preparations	1	0.11491	18.15	1	0.61959	119.11	1	0.13553	39.48
Collaborators × preparations	9	0.00463	0.73	13	0.00499	0.96	4	0.00240	0.70
Error	160	0.00633	1.00	188	0.00520	1.00	86	0.00343	1.00

Stability Observed in the Standard Deviation.—The standard deviation has been computed separately from the individual log-doses for each group of cats in Table II with the results listed in Table VII. Since here they did not increase the variability, differences between days have been disregarded in computing these basic values. The first experiment of Collaborator 6 was omitted from all means because the rate of injection differed markedly from the others. Because of their mutual consistency in respect to s^2, the variances within the two series by Collaborators 1 and 8 in the third experiment have been combined. The degrees of freedom for any individual standard deviation varies from 3 to 12, and each average is the square root of the mean square (the variance) for the given row or column. Restating the problem, did the terms given as standard deviations in Table VII vary more from one another than could be expected if they represented populations of equal variability?

Due to the unequal numbers of cats in each group, the agreement between their standard deviations was tested most conveniently by the χ^2 test for the homogeneity of the variance, described by Bartlett (1). Chi-square has been computed for each column in Table VII with the results shown at the foot of the table. In no case were the variances sufficiently diverse to be incompatible with the average for a given preparation and experiment. The average standard deviations for each collaborator for all assays varied from 0.056 to 0.087 but were based upon too few degrees of freedom to be demonstrably inconsistent with the mean value ($P = 0.14$). As data accumulate, the standard deviation in some laboratories may be stabilized at a lower level than in others, enabling them to determine potency with fewer cats for a specified precision.

The standard deviations in Table VII tended to diminish in successive assays, so that the variation between averages has been examined by χ^2 in Table VIII. The average for the four prepared tinctures, $s = 0.0756$ ($n = 350$), proved significantly larger than that based upon the three powders made freshly into tincture by the experimenter, $s = 0.0600$ ($n = 171$), as shown by the first row in the table. The remaining five comparisons between the seven preparations were grouped into (*a*) a seasonal factor which totaled the separate χ^2's comparing the first and second experiments in respect to tinctures and the second and third experiments in respect to powders, and (*b*) the remainder between the two tinctures or powders which were compared in each experiment. Neither approached significance. The final row of the table totals the χ^2's for the agreement between collaborators for each preparation; it was well within the range for homogeneity.

The individual lethal doses tended here to be less variable when determined with freshly prepared tinctures than with tinctures that have stood.

TABLE VII.—STANDARD DEVIATIONS COMPUTED FROM THE INDIVIDUAL LOG-DOSES SEPARATELY FOR EACH GROUP OF CATS IN TABLE II

Lab. No.	First Experiment		Second Experiment			Third Experiment		Average
	Tincture	85% Tincture	Tincture	80% Tincture	Powder	Internat. Std.	Powder	
1	0.1008	0.1029	0.0876	0.0685	0.0940	0.0696[b]	0.0673[b]	0.0853
2	0.0756	0.0566	0.0678	0.0517	0.0790	...	...	0.0669
3	0.0545	0.0313	0.0782	0.0896	0.0770	0.0443	0.0619	0.0616
4	0.1218	0.0954	0.0492	0.0576	0.0529	...	...	0.0804
5	0.0603	0.0765	0.0804	0.0843	0.0565	...	...	0.0718
6	0.0560[a]	0.1092[a]	0.0899	0.0858	0.0214	...	...	0.0754
7	0.0744	0.0847	...	...	...	...	...	0.0797
8	0.0487	0.0662	0.0508	0.0536	0.0705	0.0571[b]	0.0558[b]	0.0575
9	0.0500	0.0940	0.0903	0.0779	0.0623	0.0421	0.0467	0.0704
10	0.0597	0.0998	...	...	...	0.0578	0.0603	0.0754
11	....	....	0.0273	0.0672	0.0659	0.0859	0.0540	0.0631
13	....	....	0.0869	0.0942	0.0669	...	...	0.0837
14	....	....	0.0437	0.0583	0.0686	...	...	0.0558
15	....	....	0.0786	0.0467	0.0413	...	...	0.0573
16	0.0943	0.0781	...	...	...	...	...	0.0866
19	....	....	0.0542	0.0700	0.0333	...	...	0.0558
20	....	....	0.0791	0.0851	0.0442	...	...	0.0745
Av.	0.0773	0.0815	0.0711	0.0731	0.0614	0.0593	0.0580	0.07085
χ^2	14.572	12.946	15.604	7.392	16.417	4.866	1.255	22.199
n	9	9	13	13	13	5	5	16

[a] Values omitted from the averages.
[b] Degrees of freedom equal to number of cats listed in Table I diminished by 2; in all other standard deviations, the degrees of freedom are 1 less than the number of cats.

TABLE VIII.—CHI-SQUARE TESTS FOR HOMOGENEITY OF THE VARIANCES LISTED AS STANDARD DEVIATIONS IN TABLE VII

Difference in Variance Between	Degrees of Freedom	χ^2	P
All tinctures *vs.* all powders	1	11.506	<0.001
Successive experiments for tinctures and for powders	2	1.695	0.42
Paired tinctures or powders in the same experiment	3	0.319	>0.95
Collaborators within experiments and preparations	67	73.052	0.29

The apparently high significance of the first entry ($P < 0.001$) and the small size of the other χ^2's supports this conclusion. Since the different collaborators were not represented equally on both sides of the comparison, χ^2 has been recomputed from the pooled variances for tinctures and for powders using only the participants in the third experiment. Though the difference was less significant than when computed from all of the data, this selected portion showed similarly a smaller standard deviation for powders than for tinctures ($s = 0.0625$ and 0.0748, respectively).

Relation of a Stable Standard Deviation to Assay Technique.—The general agreement of the individual standard deviations with their average values suggests that with a stabilized technique the variation in the standard deviation may not exceed the limits expected by statistical theory, especially within laboratories. Such an outcome would give a distinct advantage. If the standard deviations within successive groups of cats proved mutually consistent, the true or "population" value would be approached with increasing precision. The combined standard deviation based upon past tests could then serve as a laboratory constant for increasing the precision of digitalis assays.

The reason for the increased precision is to be found in the method for computing relative potency and its error. In terms of logarithms, the relative potency of an unknown preparation (M) is computed as the difference between the mean dose of the reference standard ($\bar{y}_S$) and that of the sample or unknown ($\bar{y}_U$) or symbolically

$$M = \bar{y}_S - \bar{y}_U. \qquad (2)$$

Since 1.0 cc. of the tincture prepared from U. S. P. Standard Digitalis is equivalent to 1.0 U. S. P. Digitalis Unit, the antilogarithm of M in an assay with the Reference Standard not only shows the relative potency of the unknown but also its content of U. S. P. Digitalis Units per cc. of tincture. The error of this log-ratio of potencies, s_M, is the standard error of the difference between two means or

$$s_M = s\sqrt{\frac{1}{N_S} + \frac{1}{N_U}}. \qquad (3)$$

where N_S and N_U are the numbers of cats injected with the standard and unknown, respectively, and used in solving Eq. (2).

In computing the standard deviation, s, for Eq. (3), however, we may draw upon the data from former assays. If s is computed only from the sums of squares (see Eq. (1)) for the N_S and N_U individuals in a given assay as

$$s = \sqrt{\frac{[y^2]_S + [y^2]_U}{N_S + N_U - 2}}, \qquad (4)$$

its reliability is less than if determined from many similar experiments representing independent estimates of the same standard deviation. In the latter case the spread of the observations for a given group of cats would be checked with the aid of a control chart or its equivalent (3, 16, 17) against that expected from the standard deviation based upon 10 to 15 or more mutually consistent earlier groups. If in agreement with expectation, the standard deviation would be in a state of "statistical control" and that representing the larger experience could be used in computing the error of the assay.

To understand how this increases the precision of the assay, the calculation must be carried a step further. The best estimate from a given assay of the true relative potency of an unknown is given in logarithms by M in Eq. (2). Because of the variability in the individual lethal dose, M will seldom coincide with the true value. With s_M, however, we can compute a lower limit, such that if the true potency were equal to this lower limit a value as large as the observed M would not be expected to occur oftener than once in 40 similar assays. In the same way, we can compute an upper limit such that if in reality it were the true value, an M as small as that observed would not be expected oftener than once in 40 similar trials. Thus two boundaries can be determined, known as confidence or fiducial limits (here they are numerically identical) which the true potency of the unknown would not be expected to exceed in either a plus or minus direction oftener than once in 20 assays. These boundaries are given by the equation:

$$\text{Log (confidence limits)} = M \pm ts_M \qquad (5)$$

where t is read from a table of Student's distribution, such as Table III in reference (7), at $P = 0.05$ with as many degrees of freedom as in the standard deviation (s) used to compute s_M.

Since the concentration of an unknown preparation is adjusted to 100% potency on the basis of the assay, the value of M is made exactly equal to zero. If the adjustment is accurate, the odds are then 19 in 20 that the true but unknown log-potency of the sample falls within limits of $\pm ts_M$.

The precision of an assay, therefore, varies inversely with both s_M and t. If s in s_M is computed from the variation in a self-contained assay, t will be based upon $N_S + N_U - 2$ degrees of freedom. For an assay with six cats on the standard and six cats on the unknown, the value of t at $n = 10$ and $P = 0.05$ is 2.228. If the observed s for the single assay is consistent with a "laboratory s" based upon many assays, representing, say, 120 degrees of freedom, and is replaced by this more general value, t is reduced from 2.228 to 1.980 with a consequent narrowing of the confidence limits. Thus the utilization of consistent past experience of this extent would increase the precision as much as if the assay were run with 15 cats instead of 12.

The median lethal dose for the Reference Standard also may vary only within its sampling error in successive experiments within a given laboratory. The mean log-dose observed in a single assay could then be checked against this accumulated experience. If consistent with the assumption of a stable susceptibility, it could be replaced by the laboratory mean in computing M. In calculating the standard error of M from Eq. (3), $1/N_S$ would no longer be approximately equal to $1/N_U$ but a progressively smaller fraction with successive assays, so that s_M would approach a value 71% as large as in a laboratory where the lethal dose does not have the requisite stability. Thus narrower confidence limits would be gained without an increase in the number of cats in an individual assay.

Calculation in Original Units.—The conversion of individual lethal doses in Table II to logarithms made it possible to study the standard deviation independently of the mean. A sound program for utilizing the accumulated experience of a laboratory would also be based upon the log-dose. An isolated, self-contained assay, on the other hand, can be computed with nearly equal validity from either logarithmic or original units. In its instructions for calculating the results of a digitalis assay, the U. S. P. XII uses original units instead of logarithms. Since the official computation is in original units, its form is of some interest.

The procedure differs from that described for logarithms primarily in the substitution of ratios for differences. The average lethal dose for the Reference Standard ($\bar{c}_S$) is divided by that for the sample or unknown ($\bar{c}_U$) to obtain the potency of the sample in terms of U. S. P. Digitalis Units (D) per cc. of tincture.

$$D = \frac{\bar{c}_S}{\bar{c}_U} \qquad (6)$$

The standard error of each mean ($\bar{c}$) in this ratio is computed directly from the corresponding lethal doses (c) for the individual cats, N in number, as

$$\sqrt{\frac{S(c - \bar{c})^2}{N(N-1)}}$$

which is expressed as a ratio to the mean of its group by the equation

$$E = \frac{1}{\bar{c}}\sqrt{\frac{S(c - \bar{c})^2}{N(N-1)}}. \qquad (7)$$

The group on the standard may be distinguished from that on the unknown by subscripts, as E_S and E_U. The Pharmacopœia requires that if the standard error of an average lethal dose, either for standard or for unknown, exceeds 5.7%, the group is to be repeated as a whole or enlarged by injecting additional cats until E is equal to or less than 0.057. Since the two groups are independent of each other, in the sense that the standard does not determine experimentally the lethal dose for the unknown and vice versa, the standard error of potency in U. S. P. units of digitalis (s_D) may be written

$$s_D = D\sqrt{E_S^2 + E_U^2}. \qquad (8)$$

Note that its standard error varies directly with the potency, D. This is also true of the confidence or fiducial limits computed from s_D, since in terms of U. S. P. Digitalis Units per cc.,

$$\text{Average confidence limits} = D \pm ts_D. \qquad (9)$$

In the U. S. P. XII, the potency of a sample of digitalis is determined from a self-contained assay and when reassayed it must fall within tolerance limits of 0.8 and 1.2 units per cc. of tincture. The range of potencies indicated by the confidence or fiducial limits of an official assay, at odds of 19 in 20, should be less than the required tolerance. For an assay with the specified minimum of six cats on the standard and six cats on the unknown, $t = 2.228$ at $P = 0.05$ and $n = 10$. If the standard error of the average dose for both groups of cats just met the requirement of $E = 0.057$, the average confidence limits by Eqs. (8) and (9) would be $D \pm 2.228D\sqrt{0.057^2 + 0.057^2} = D \pm 0.180D$. Nineteen out of twenty preparations adjusted on the basis of such an assay to the standard potency of $D = 1.0$ U. S. P. Digitalis Units per cc. of tincture would be comfortably within the specified tolerance.

Meeting the U. S. P. Tolerance.—The first U. S. P. XII Bound Supplement provides that tincture of Digitalis "shall be considered to conform to the pharmacopœial requirement if the result of the assay . . . does not vary more than 20 per cent from such requirement." Hence the range of acceptance in terms of M is from the logarithm of 0.80 or -0.0969 to the logarithm of 1.20 or 0.0792.

After preparations have been adjusted on the basis of the original assay to 100% potency or $M = 0$, they could still fail to meet this specification oftener than once in 20 check assays, despite confidence limits from Eq. (5) equal to the tolerance at $P = 0.05$. They would pass if each had a true potency of exactly 100%, but the potency from bioassay seldom agrees so precisely with the real value, as will be shown in Section IV. Hence the true potency of a preparation usually falls somewhat above or below claim but in 19 cases out of 20 within the confidence limits of $\pm ts_M$. Within this range, the greater the departure from claim ($M = 0$) the greater are the odds that the check assay of a governmental control agency will show a potency outside the tolerance. Hence if the confidence limits of the original assay are equal to the U. S. P. tolerance, the potency of samples adjusted to 100% on this basis will vary over too wide a range to pass a later inspection at the same odds. For assurance that not more than one batch in 20, let us say, will fail at inspection, the manufacturer may prefer to narrow the confidence limits of his assays. How can one determine the number of cats which will reduce the error sufficiently without waste of animals?

For a satisfactory solution the standard deviation must be stable in the successive assays of a given laboratory, so that it represents many degrees of freedom and has predictive value. Moreover, the standard deviation in the control laboratory should also be in statistical control and its value known. Then the required confidence limits are those for the difference between two log-ratios of potency, that for the original assay (M_1) and that for the check assay (M_2). This depends upon the standard error of the difference between two differences or

$$s_{M_2 - M_1} = \pm\sqrt{s_2^2\left(\frac{1}{N_S} + \frac{1}{N_U}\right)_2 + s_1^2\left(\frac{1}{N_S} + \frac{1}{N_U}\right)_1} \qquad (10)$$

where subscripts 1 and 2 indicate the original and the check assays, respectively. While this is the error of the difference $M_2 - M_1$, it is also the predicted error of $M_2 - 0 = M_2$, since M_1 is made equal to zero in adjusting the potency of the preparation to the pharmacopœial requirement. For acceptance M_2 must not exceed specification limits of -0.0969 and $+0.0792$. Hence the required confidence limits, computed with the standard error in Eq. (10), are

$$-0.0969 \leqslant t\,s_{M_2 - M_1} \leqslant 0.0792 \qquad (11)$$

where t is read from a table of t (7) at $P = 0.05$ for odds of passing 19 in 20 preparations. In demonstrating their stability, s_1 and s_2 probably will have been determined with 120 or more degrees of freedom, so that t may be taken as 1.980 for this level of security. For greater certainty, such as odds of 99 in 100, t would be increased to 2.617.

How many cats should be injected in the manufacturer's assay to meet the requirement in Eq. (11)? For a correct answer all terms in Eq. (10) should be known, other than N_S and N_U in the original assay. Since they are not known, we will assume here that $N_S = N_U = N$ and that $s_1 = s_2 = s$. Then Eqs. (10) and (11) may be combined and simplified to the form

$$-0.0969 \leqslant ts\sqrt{\frac{2}{N_1} + \frac{2}{N_2}} \leqslant 0.0792. \qquad (12)$$

With $s = 0.0756$, the combined standard deviations for tinctures in the first and second collaborative experiments, and with $t = 1.980$, Eq. (12) reduces to

$$-0.0969 \leqslant 0.1497\sqrt{\frac{2}{N_1} + \frac{2}{N_2}} \leqslant 0.0792.$$

When solved for N_1 on the assumption that $N_2 = 6$ in the control assay, the lower specification limit would require that 24 cats ($N_1 = 23.3$) be run on the standard and 24 on the sample, while the upper limit could be met only if the standard deviation were less than 0.0756. It seems probable, however, that a control agency would run two or more U. S. P. assays on any doubtful preparation, in which case there would be 12 cats on the standard and 12 cats on the sample (N_2). When solved for $N_2 = 12$, the lower tolerance would be met by $N_1 = 8$ and the upper limit by $N_1 = 10$ or 11 animals ($N_1 = 10.2$ cats). Since the limit applying to any given sample cannot be predicted, 10 or 11 cats would be required for both standard and unknown if the values assumed for t, s_1 and N_2 were applicable.

The above calculation requires that only the

standard deviation shall be in a state of statistical control, a requirement well within reach if one may judge from the collaborative assays. In some laboratories the mean log-dose ($\bar{y}_S$) for the reference standard may also be stabilized. The data from cats injected with the reference standard in any given assay could then be augmented by all previous records of the same laboratory, and $1/N_S$ in Eq. (10) would become a relatively small number in comparison with $1/N_U$. Potency would then be established within narrower confidence limits without an increase in the number of cats.

IV. RELATIVE POTENCIES IN THE COLLABORATIVE ASSAYS

Having studied the mean and standard deviation separately, our next task is to see how they worked together in the 47 individual assays of the collaborative experiments. The problem has two parts. The first is the relation between sample and population or the precision of the assay. When several independent samples are available in a given series, such as the separate assays reported by different collaborators, we can determine the probability that they represent the same population. If the variation between samples would be expected from the variation within samples, they can be considered as homogeneous. The potency computed from the entire series then provides a more precise estimate of the unknown population than any single assay.

The second part is the relation between the population and the true value or the accuracy of the assay. Have our samples been drawn from the population in which we are interested? If the true potency of the "unknown" sample is known, independently of biological assay, the accuracy as well as the precision of the assay can be determined. Since this occurred in the three comparisons of the first two collaborative experiments, the assayed potency and the statistical estimate of its precision could be compared with the true potency both for the means and for the individual assays of each series.

Homogeneity and Precision of Replicated Assays.—In considering a replicated series of assays, two questions may be asked: (*a*) Do the individual assays agree with one another, and (*b*) what potency falling within what limits is indicated by the series as a whole? The mutual consistency of the potencies reported in three of the four series from the collaborative assays has already been tested by the analyses of variance in Table VI. These showed good agreement as to the relative potencies of the dilute tinctures in the first two experiments and of New Study Powder compared with International Standard Digitalis (1936) in the third experiment. The series in the second experiment comparing the potency of powder relative to tincture has not been examined. Moreover, all estimates of the variance from individual assays were pooled in the analysis of variance for each of the three series. Could the same conclusion be reached by a different mode of calculation, preferably one which would lead directly to an estimate of potency?

An alternative test for homogeneity is based on the χ^2 distribution and the mean log-potency for the series. It depends upon the standard errors of the individual assays and has the advantage that these errors can be computed with either pooled or individual estimates of the variance. In either case, the log-potency, M, is calculated as the difference between the two mean log-doses for each assay by Eq.

TABLE IX.—THE LOG-RATIO OF POTENCY (M) FOR INDIVIDUAL ASSAYS, COMPUTED FROM THE DATA IN TABLE II

The first set of weights assigned each assay (W_1) have been computed with the pooled variances (s_1^2) in the last row, the second series of weights (W_2) with the variances for the separate assays. The means (M) and χ^2's computed by each method are identified by subscripts 1 and 2, n giving the degrees of freedom.

Lab. No.	First Experiment, 85% Tincture M	W_1	W_2	Second Experiment, 80% Tincture M	W_1	W_2	Second Experiment, Powder M	W_1	W_2	Third Experiment, Powder M	W_1	W_2
1	−0.0649	713	434	−0.1105	577	485	−0.0740	671	363	0.0122	872	640
	....	..	..	....	..	..	....	..	..	0.0900	727	533
2	0.0074	713	1009	−0.1272	577	826	−0.0227	671	554	....	..	..
3	−0.0384	713	2278	−0.0510	577	424	0.0538	671	498	0.1008	1308	1553
4	−0.0848	713	376	−0.0524	989	1836	0.0678	895	1575	....	..	..
5	−0.1089	713	948	−0.1165	577	442	−0.0390	671	621	....	..	..
6	−0.0445[a]	671	541	−0.1655	718	485	−0.1710	723	700	....	..	..
7	−0.0370	713	708	....	..	..	....	..	..	....	..	..
8	−0.0250	751	1427	−0.1095	577	1101	−0.0130	671	795	0.0718	872	942
	....	..	..	....	..	..	....	..	..	0.0543	872	942
9	−0.0726	751	809	−0.0860	1145	827	0.0010	1333	958	0.0912	1453	2532
10	−0.0428	713	666	....	..	..	....	..	..	0.1199	697	680
11	....	..	..	−0.0862	577	1142	−0.0123	671	1179	0.0847	872	583
13	....	..	..	−0.1306	692	430	0.0520	767	595	....	..	..
14	....	..	..	−0.0691	757	1547	−0.0140	806	1207	..	..	..
15	....	..	..	−0.1572	621	808	−0.0480	671	761	....	..	..
16	−0.0477	713	601	....	..	..	....	..	..	....	..	..
19	....	..	..	−0.1322	1049	1358	0.0429	1119	2472	..	..	..
20	....	..	..	−0.1314	854	664	−0.0738	839	794	....	..	..
$\bar{M}_1$	−0.0514 ± 0.0118			−0.1076 ± 0.0099			−0.0135 ± 0.0168			0.0792 ± 0.0114		
$\bar{M}_2$	−0.0455 ± 0.0120			−0.1014 ± 0.0107			−0.0037 ± 0.0197			0.0824 ± 0.0136		
True M	−0.0706			−0.0969			0					
χ_1^2	6.943, n = 9			12.474, n = 13			40.833, n = 13			6.589, n = 7		
χ_2^2	8.748, n = 9			15.090, n = 13			44.969, n = 13			5.716, n = 7		
s_1^2	0.006309, n = 162			0.005202, n = 188			0.004469, n = 176			0.003441, n = 90		

[a] Assay omitted from means, χ^2's and s_1^2.

(2) with an error, s_M, as given by Eq. (3). Each M is assigned a weight, W, based upon this error by the equation

$$W = \frac{1}{s_M^2}. \qquad (13)$$

Using these weights, the mean log-potency for the series is

$$\overline{M} = \frac{S(WM)}{S(W)}. \qquad (14)$$

The weighted sum of squares of the k individual assays about this mean is distributed as χ^2 with $k - 1$ degrees of freedom or

$$\chi^2 = S(WM^2) - \overline{M}S(WM). \qquad (15)$$

χ^2 may be used both in selecting the better method for estimating the standard deviation of the individual assay and for testing the homogeneity of the assays in a series.

The standard error of M, s_M, may be calculated by Eq. (3) with one of two different values for the standard deviation. The first is a single standard deviation used for all replicates, on the assumption that differences in s^2 between assays are due to the errors of random sampling from the same population. In each series of the collaborative experiments the pooled standard deviations have been computed from mean squares with 90 to 188 degrees of freedom based upon all component assays. The homogeneity of the variances observed in Section III is decisive in support of this first approach. Alternatively, every s_M may be computed with a different standard deviation, determined in each case from the data of the individual assay. The true standard deviation is then assumed to differ from one laboratory to another and any appearance of homogeneity between samples is attributed to their small size. In accord with this second, less tenable assumption, each s_M was recomputed with the standard deviation for the individual assay, representing 10 to 22 degrees of freedom.

The individual log-ratios of potency (M) are given in Table IX together with the weights assigned to each when the latter were computed with the pooled s_1^2 for the series (W_1) and with the s^2 from the separate assays (W_2). The contributions of the individual M's to the mean were more nearly equal by the first than by the second method, so that the resulting means, $\overline{M}_1$ and $\overline{M}_2$, were not identical. If the individual variances reflected real differences in the standard deviations, the χ_2^2's which measure the variation about M_2 should be consistently less than the χ_1^2's which measure the variation about each M_1. The reverse was true in three of the four comparisons (Table IX), smaller values of χ^2 resulting with the first series of weights (W_1). Hence on the basis of this supplementary test of relative homogeneity, it would be preferable to compute s_M for the individual assays with pooled estimates of the standard deviation.

The χ^2's also tested the homogeneity of the individual assays in a series. In comparisons of two prepared tinctures or of two powders where both were made into tinctures by each collaborator (the third experiment), there was good agreement between laboratories with either method of weighting. However, the comparison of "powder" with a prepared tincture in the second experiment gave inconsistent results, the χ^2's showing a highly significant heterogeneity between collaborators ($P < 0.001$). This was due largely but not entirely to a single "bad" assay, that for Collaborator 6. The omission of this value reduced χ^2 substantially, to $\chi_1^2 = 21.702$ and $\chi_2^2 = 24.274$ ($n = 12$), values which still exceeded the limits usually accepted for a homogeneous series.

The problem remains of determining the precision of the weighted mean log-potency as computed from replicated assays by Eq. (14). The precision may be expressed in terms of the standard error of $\overline{M}$, which depends upon both the variance, s_M^2, of each component and the variation between the M's, such as is measured by χ^2 in Eq. (15). The latter term is usually neglected if χ^2 indicates good agreement between individual assays. Where the standard deviation is stable as well, so that a single, pooled s^2 can be used in computing the s_M^2 for each assay, the standard error of the mean, $\overline{M}$, may be calculated as

$$s_{\overline{M}} = \sqrt{\frac{1}{S(W)}}. \qquad (16)$$

The standard errors of $\overline{M}$ for the three homogeneous series in Table IX were determined in this way.

If the standard deviations in the separate assays were known or assumed to differ, the errors of the individual values (s_M) would be computed with their respective standard deviations. Under these conditions, Cochran (5) has shown that equations such as Eq. (16) underestimate the error of the weighted mean. He found that most of this bias could be corrected by multiplying by $\sqrt{n/(n-4)}$, with n equal to the degrees of freedom in the individual variances. Where n varies as here from one assay to another, the average degrees of freedom may be used as a first approximation, giving

$$s_{\overline{M}} = \sqrt{\frac{\overline{n}}{S(W)\,(\overline{n} - 4)}}. \qquad (17)$$

The standard errors of M_2 for the homogeneous series in Table IX have been computed by Eq. (17) and in every case the precision of M_2 proved less than that of $\overline{M}_1$.

Where the individual M's, k in number, do not agree with one another by the χ^2 test, the variation between them cannot be neglected and the reliability of their mean is reduced proportionately. In this case the values of the standard error given by Eqs. (16) and (17) may be multiplied by

$$\sqrt{\frac{\chi^2}{k - 1}}$$

to correct the discrepancy. The standard errors for the mean log-potencies of the powder in the second experiment (Table IX) were computed with this adjustment.

Comparison of Assayed and True Potencies.— The mean log-potency of each comparison in the collaborative assays represented a more precise estimate than would ordinarily be available. Since the true value was known experimentally in the three comparisons of the first two experiments (Table IX), the cat assay could be examined with reference to its accuracy as well as its precision. None of the mean log-potencies computed with pooled estimates of the standard deviations ($\overline{M}_1$) differed more from the true M than could be expected from their respective errors, one falling above and two below the correct value. When the standard deviations were not pooled, $\overline{M}_2$ for the first experiment was significantly greater than the true value of -0.0706, with $t = 2.096$ and $n = 162$. Evidently the variation in s from one collaborator to another in the first experiment did not reflect real differences in relative precision but fortuitous variability, which here gave the larger weights to the less reliable assays.

Hence upon the grounds of accuracy as well as precision, standard errors and weights based upon the pooled s^2 would be preferred.

In the assay of powder *vs.* tincture in the second experiment, the marked heterogeneity introduced by the result from Collaborator 6 has already been noted. The omission of this value greatly reduced the χ^2 for the restricted series and at the same time brought its mean nearer to the true value of zero from $\overline{M}_1 = -0.0135 \pm 0.0168$ to $\overline{M}_1 = -0.0026 \pm 0.0131$. The remaining heterogeneity ($P = 0.04$) was not sufficient to justify any further omissions.

Potency is usually determined from a limited number of assays, only one being required by the Pharmacopœia. While the mean of a long series can give both a precise and an accurate result, the distribution of the component tests about the true value indicates what may be expected from individual assays. The M's in Table IX have been plotted in the frequency distributions of Fig. 3.

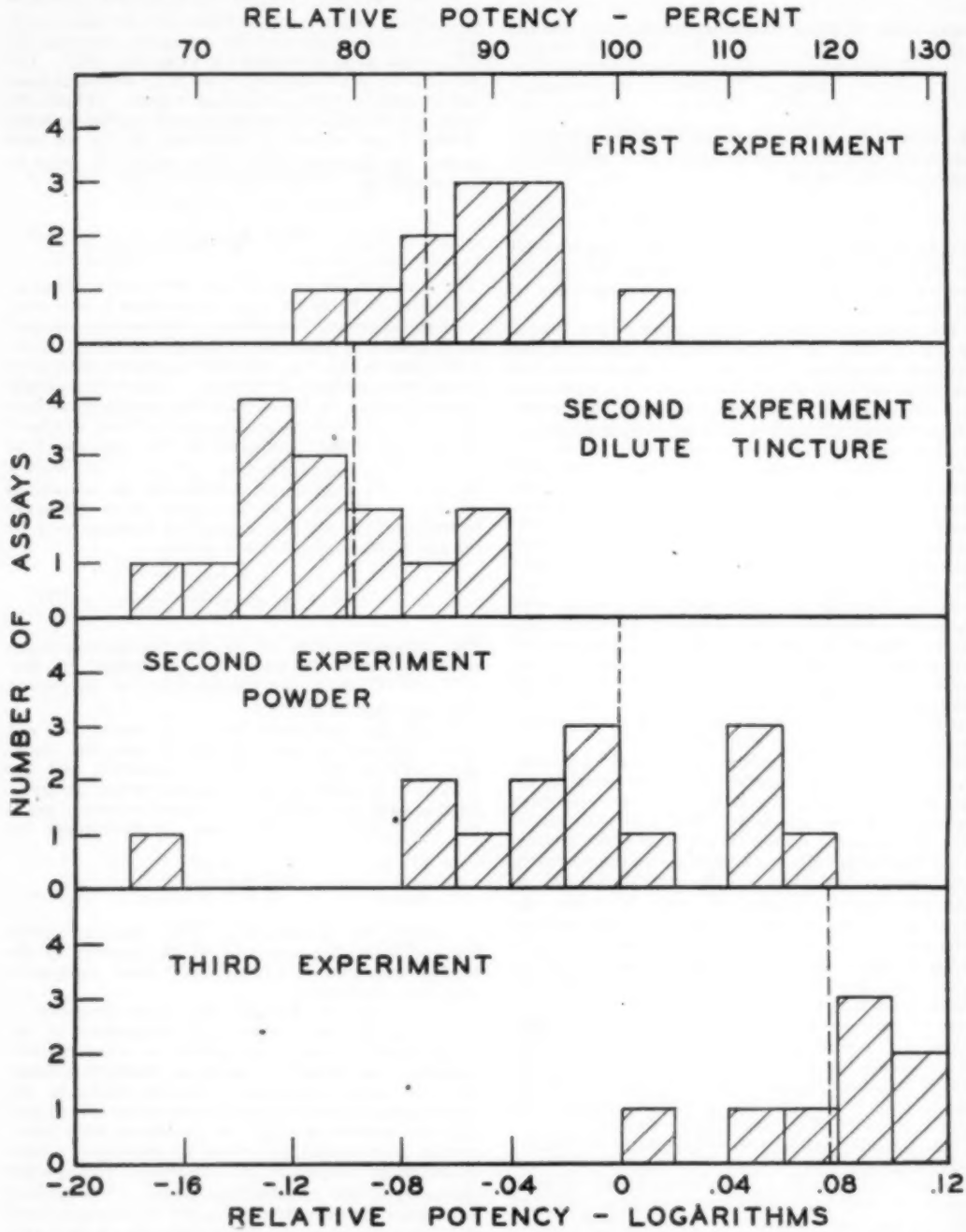

Fig. 3.—Frequency distributions of the relative potencies observed in the individual assays. The broken vertical lines in the first and second experiments are the true potencies and in the third experiment the value used in preparing U. S. P. Digitalis Reference Standard from New Study Powder.

The broken vertical lines represent the true values, except in the third experiment, where it is the factor used in preparing U. S. P. Digitalis Reference Standard (1942) from the New Study Powder.

In Table X the potencies have been listed in original units with their standard errors. Only two of the 39 assays in the first two experiments gave the correct answer to the second decimal place, although, with one exception, they were distributed no more widely than would be expected statistically. This could be checked by comparing each log-potency with the correct value. The true M was subtracted from each observed M in Table IX and the difference divided by s_M to obtain the statistic t. Table X shows from the t for each assay the probability P of a departure from the true value as large as or larger than that observed. In a series of 38 acceptable assays, a probability less than 0.05 would be expected in about two cases; three were found. The distribution of these probabilities supfound potencies which did *not* diverge significantly from 100%.

The second experiment also compared prepared 100% tincture against an equivalent tincture made by each collaborator from the same New Study Powder. If the latter were made uniformly, the individual potencies should not differ significantly from 100%. Three of the 14 assays diverged from the true value more than would be expected by chance in isolated assays. Even in a shorter series, No. 6 would be rejected by the χ^2 test for homogeneity; in one of the other two assays (No. 4) "powder" tested too high and in the other (No. 20) too low. The good agreement between tinctures in the same experiment suggests discrepancies in the preparations of tincture from powder rather than in the assay procedure.

Upon completion of the third experiment, the data were analyzed by Dr. Lloyd C. Miller by a slightly different method. He estimated that 1 cc.

TABLE X.—RELATIVE POTENCIES OBSERVED IN INDIVIDUAL ASSAYS WITH STANDARD ERRORS BASED UPON POOLED ESTIMATES OF s^2

The probabilities (P) give the odds of obtaining a discrepancy from the true value as great as or greater than that observed. Both statistics are determined from the data in Table IX.

Lab. No.	First Experiment: 85% Tincture Potency	First Experiment: 85% Tincture P	Second Experiment: 80% Tincture Potency	Second Experiment: 80% Tincture P	Second Experiment: Powder *vs.* Tincture Potency	Second Experiment: Powder *vs.* Tincture P	Third Experiment: Powder *vs.* I. S. D. Potency
1	0.86 ± 0.07	0.88	0.78 ± 0.07	0.75	0.84 ± 0.08	0.06	1.03 ± 0.08
	.....	...	.....	..	.....	...	1.23 ± 0.11
2	1.02 ± 0.09	0.041	0.75 ± 0.07	0.47	0.95 ± 0.08	0.56	.....
3	0.92 ± 0.08	0.25	0.89 ± 0.09	0.27	1.13 ± 0.10	0.17	1.26 ± 0.08
4	0.82 ± 0.07	0.71	0.89 ± 0.06	0.17	1.17 ± 0.09	0.046	.....
5	0.78 ± 0.07	0.31	0.76 ± 0.07	0.64	0.91 ± 0.08	0.31	.....
6	0.90 ± 0.08[b]	0.50	0.68 ± 0.06	0.07	0.67 ± 0.06[b]	<0.001	.....
7	0.92 ± 0.08	0.37	.....	..	.....	...	.....
8	0.94 ± 0.08	0.22	0.78 ± 0.07	0.76	0.97 ± 0.09	0.74	1.18 ± 0.09
	.....	...	.....	..	.....	...	1.13 ± 0.09
9	0.85 ± 0.07	0.94	0.82 ± 0.06	0.71	1.00 ± 0.06	0.97	1.23 ± 0.07
10	0.93 ± 0.08	0.46	.....	..	.....	...	1.32 ± 0.11
11	.....	...	0.82 ± 0.08	0.80	0.97 ± 0.09	0.75	1.22 ± 0.09
13	.....	...	0.74 ± 0.06	0.38	1.13 ± 0.09	0.16	.....
14	.....	...	0.85 ± 0.07	0.45	0.97 ± 0.08	0.69	.....
15	.....	...	0.70 ± 0.06	0.14	0.90 ± 0.08	0.22	.....
16	0.90 ± 0.08	0.54	.....	..	.....	...	.....
19	.....	...	0.74 ± 0.05	0.26	1.10 ± 0.08	0.16	.....
20	.....	...	0.74 ± 0.06	0.32	0.84 ± 0.07	0.037	.....
Mean	0.888 ± 0.024	0.11	0.781 ± 0.018	0.32	0.994 ± 0.030	0.84	1.200 ± 0.032
True value	0.85		0.80		1.00		1.194[a]

[a] Factor used subsequently in converting New Study Powder to U. S. P. Digitalis Reference Standard.
[b] Omitted from means.

ports the validity of the conclusions in Section III based upon confidence or fiducial limits.

The effectiveness of the individual assays in Tables IX and X is worth reviewing. In the first experiment only one biological determination differed significantly ($P < 0.05$) from the concentration known by dilution, the potency of 102% reported by Collaborator 2. Conversely, only Collaborator 5 showed a significant discrepancy between the 100 and 85% tinctures, in agreement with the analyses of variance in Table III. Although nine of the eleven collaborators, including No. 6, reported potencies nearer to 85 than to 100%, an individual assay with nine cats on the standard and nine cats on the unknown had here too little power to recognize a difference of 15%. The dilute tincture or unknown in the second experiment was 80% of full strength. The instructions called for a minimum of six cats on each preparation, but eight of the 14 collaborators used more. In this comparison no one reported a value diverging significantlp from 80% and only Collaborators 3 and 4 of tincture prepared from New Study Powder represented a potency equal to 1.194 ± 0.033 International Digitalis Units. Hence 194 Gm. of exhausted marc were added to each 1000 Gm. of New Study Powder and the diluted preparation became the U. S. P. Digitalis Reference Standard, containing the equivalent of 10 International Units in each gram by the U. S. P. XII cat assay. The estimated mean $\bar{M}_1$ obtained with W_1 differs from this value by less than one-fifth of its error of estimate. Such close correspondence, coupled with the good agreement between laboratories in the third experiment in absolute as well as relative toxicities (Table VI), indicates that the intended equivalence of the U. S. P. and International Digitalis Units has been achieved.

V. SUMMARY

This paper describes the collaborative assays of digitalis upon which the mono-

graph in the U. S. P. XII is based. Sixteen collaborators submitted data in three experiments upon the New Study Powder, representing a total of 616 cats. The first experiment compared a prepared tincture and an 85% concentration of the same tincture. The second experiment compared a prepared tincture, an 80% concentration of the same preparation and tinctures freshly prepared by each collaborator from the original powder. Since the exact potency was known in the comparisons of the first two experiments, though not disclosed in advance, both the precision and the accuracy of the assay technique could be determined. The third experiment compared the International Standard Digitalis (1936) and the New Study Powder to obtain the factor used in preparing the U. S. P. Digitalis Reference Standard from the latter, so that one U. S. P. Digitalis Unit would have the same activity by the U. S. P. cat assay as one International Digitalis Unit.

The first problem in analyzing these data statistically was to convert them to units which would be normally distributed. The log-dose filled this need and has been used through most of the paper. It was then possible to study the stability of the mean susceptibility to digitalis independently of the variation between the cats within a group. Changes in response due to differences in laboratory technique were here indistinguishable from those due to differences in the inherent resistance of the cats to poisoning by digitalis; both factors are included in the term susceptibility. Over short periods, of fifteen days or less, there was no more variation between the cats tested in the same laboratory with the same preparation than if they had all been injected on the same day. However, in intervals between successive experiments of seven to ten weeks, three weeks in one case, the susceptibility changed significantly in several laboratories. Even greater differences occurred between collaborators. The so-called "cat unit" was clearly too unstable to use for standardizing the potency of digitalis preparations. Nevertheless, collaborators agreed very well as to relative potency, when one preparation was compared with another. The only exceptions to this consistency occurred in the second experiment when each collaborator made his own tincture for comparison with a prepared tincture from the same powder. Here the discrepancies may have been due to uneven fresh extraction of the powder rather than to the assay. The approximate median lethal dose of U. S. P. Digitalis Reference Standard (the LD_{50}), of use in preparing dilutions for injection, was computed as 0.77 ± 0.09 U. S. P. Digitalis Units.

In comparison with its error, the standard deviation measuring the variation between replicate cats was more stable than the mean. Only one factor caused a significant change; the standard deviation seemed to be smaller when tinctures were freshly prepared before injection. The standard deviation probably can be stabilized, at least within a laboratory. In this case, past experience could be used in evaluating current assays with as much as a 25% increase in precision. The value of isolated, self-contained assays is limited, even if they were to give preparations meeting the specification limits of $\pm 20\%$ in 19 cases out of 20. When retested, check assays would find more than one such preparation in 20 outside the tolerance. To meet the U. S. P. requirement without an excessive use of cats, the standard deviation must be in a state of statistical control. Equations for computing the number which should be run are given and have been solved with the average standard deviation observed for prepared tinctures. On the assumption that two U. S. P. minimal assays would be used in checking any doubtful sample, 10 or 11 cats would be needed on both standard and sample for odds of 19 in 20 of passing the tolerance.

The collaborative experiments were unique in that the true answer was known in three of the four comparisons. When the relative potencies were combined, weighting each on the basis of the number of cats but pooling all estimates of the standard deviation in a series, no mean potency differed more from the true value than would be expected from the experimental error. Hence the cat assay seems to be unbiased. Comparing assayed and true values, most individual assays could not discriminate a 15% difference in potency but did differentiate a 20% difference. The accuracy of the first two experiments, where the results could be checked independently, probably carried over to the third experiment, which showed additional evidence of stability. Hence there was good reason for believing that the objective of making the U. S. P. Digitalis Unit equal in potency to the International Unit (1936) has been achieved.

All steps in the above argument are supported in the text by suitable statistical evidence. Especially where they depart from standard procedures, the equations are given and explained. The statistics used most frequently have been the analysis of variance, various forms of the χ^2 test for homogeneity and the distribution of t.

REFERENCES

(1) Bartlett, M. S., *Suppl. J. Roy. Stat. Soc.*, 4 (1937), 137.
(2) Bliss, C. I., THIS JOURNAL, 29 (1940), 465.
(3) Bliss, C. I., and Hanson, J. C., *Ibid.*, 28 (1939), 521.
(4) Burn, J. H., "Biological Standardization," Oxford Univ. Press, London, 1937.
(5) Cochran, W. G., *Suppl. J. Roy. Stat. Soc.*, 4 (1937), 102.
(6) Fisher, R. A., "Statistical Methods for Research Workers," eighth edition, Oliver and Boyd, London, 1941.
(7) Fisher, R. A., and Yates, F., "Statistical Tables for Biological, Agricultural and Medical Research," second edition, Oliver and Boyd, London, 1943.
(8) Gold, H., Cattell, McK., Kwit, N. T., and Kramer, M. D., *J. Pharmacol.*, 73 (1941), 212.
(9) Gold, H., Cattell, McK., Otto, H. L., Kwit, N. T., and Kramer, M. D., *Ibid.*, 75 (1942), 196.
(10) Hatcher, R. A., and Brody, J. G., *Am. J. Pharm.*, 82 (1910), 360.
(11) Knaffl-Lenz, E., *J. Pharmacol.*, 29 (1926), 407.
(12) Miller, L. C., THIS JOURNAL, 33 (1944), 245.
(13) Miller, L. C., Bliss, C. I., and Braun, H. A., *Ibid.*, 28 (1939), 644.
(14) U. S. P. Minutes of Conference on Digitalis Assay, Sept. 6, 1941, Hotel Pennsylvania, New York City.
(15) van Esveld, L. W., *Arch. intern. pharmacodynamie*, 45 (1941), 216.
(16) Various authors, Am. Standards Assn., "American Defense Emergency Standard," Z1.1-Z1.2 (1941).
(17) Various authors, Am. Standards Assn., "American War Standard," Z1.3 (1942).
(18) Vos, B. J., and Dawson, W. T., *J. Pharmacol.*, 57, (1936), 145.
(19) Yates, F., *J. Agr. Sci.*, 23 (1933), 108.

The U. S. P. Collaborative Digitalis Study Using Frogs (1939–1941)*

By Lloyd C. Miller†

HISTORICAL BACKGROUND

Although pharmacological activity has been recognized in digitalis for more than a century and a half, the biological assay of this important drug dates back little more than six decades. The U. S. P. IX, which became effective in 1916, was the first official compendium to supply a method of biological assay. The U. S. P. IX method used frogs as the test animal and allowed one hour for absorption of the injected digitalis. The two succeeding revisions of the U. S. P. retained the same procedure.

During the time the 1-hour method was official, there existed strong advocacy of two other methods, namely, the so-called "cat" and "overnight frog" methods. A rather extensive literature accumulated both here and abroad on the relative merits of the various techniques. Prior to 1939, several collaborative studies had been directed toward supplying data on the fundamental questions involved. For various reasons, among them the lack of objective means of evaluating the results, these early studies did not lead to unequivocal conclusions.

In 1939 the coming revision of the U. S. Pharmacopœia emphasized the need for further study and the progress that had been made in applying modern statistical methods to the digitalis assay offered a means of reaching objective conclusions. Under the sponsorship of the U. S. P. Committee of Revision plans were laid (2, 3) for the present U. S. P. Digitalis Study. Initiated in 1939, this was directed primarily toward developing evidence on which to base the assay procedure in U. S. P. XII. A secondary goal was the compositing and standardization of a digitalis powder to replace the U. S. P. Reference Digitalis Powder first released in 1936 as the official reference standard for

* Received May 23, 1944, from Winthrop Chemical Company Inc., Rensselaer, New York.

This report has been compiled at the request of the "steering committee" for the study which was composed of the chairman, Dr. E. E. Nelson, Tulane University, School of Medicine; Dr. C. W. Chapman, University of Maryland, School of Pharmacy, Baltimore, Md.; Prof. E. F. Cook, Philadelphia College of Pharmacy, Philadelphia, Pa.; Dr. J. W. E. Harrisson, La Wall and Harrisson, Philadelphia, Pa.; Dr. M. R. Thompson, Warner. Institute for Therapeutic Research, New York, N. Y.; and the author. Dr. C. I. Bliss rendered invaluable aid throughout this study and particularly in regard to the statistical treatment of the data in this report. A progress report was published earlier (1).

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U. S. P. digitalis. The study was organized in a series of related comparisons, each planned on the basis of past experience to yield data on specific questions. Throughout the study the individual assays were planned as self-contained units each representing a practical routine control potency determination.

In the course of this study results were reported on over 16,000 frogs. It is impossible either to publish the initial data in full or to discuss in detail the results of each collaborator. The original reports, the calculations and many detailed tabulations are on file in the office of the Chairman of the U. S. P. Committee of Revision. This report covers all data that were submitted, although some assays were clearly preliminary or inconclusive. The inclusion of the latter does not mean that the collaborators considered them otherwise since it was specifically directed that all pertinent data be submitted.

The following institutions or firms participated actively in the study: Department of Pharmacology, University of Maryland, School of Pharmacy; Philadelphia College of Pharmacy and Science; Department of Physiology and Pharmacology, University of Nebraska School of Pharmacy; Department of Pharmacology, Tulane University, School of Medicine; Division of Pharmacology, U. S. Food and Drug Administration; Laboratory of Hygiene, Department of Pensions and National Health of Canada; Abbot Laboratories; Applied Research Laboratory; Burroughs Wellcome and Co.; La Wall and Harrisson; Eli Lilly and Company Research Laboratory; Parke, Davis and Company; Sharp and Dohme; E. R. Squibb and Sons; Upjohn Co.; and Warner Institute for Therapeutic Research. One of the collaborators acknowledged the support by grants from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry, The American Medical Association, and from the Nebraska Foundation through the aid of the Bankers Life Insurance Company, Miller and Paine, the First National Bank, the Midwest Life Insurance Company, the Nebraska State Journal and the Star, Gold and Company, and the Lincoln Drug Company, all of Lincoln, Neb.

THE FIRST COMPARISON

General Plan.—The First Comparison of the study, started in December, 1939, and completed in the following three months, concerned the relative merits of the 1-hour and 18-hour frog methods. Two samples of powdered digitalis were provided, one of which (No. 1) was a dilution of the other (No. 2), the diluent being thoroughly exhausted marc, the inactivity of which had been proved biologically. Ten laboratories participated and each was requested to determine the relative potency of the two samples in accordance with the directions given below.

ASSAY DIRECTIONS

The essential details of the assay directions as distributed (4) are given below.

(*A*) *Preparation of a Liquid Extract (Macerate) by Maceration.*—The method of the preparation of liquid extracts (macerates) shall be as follows:

1. Use Pyrex, glass-stoppered containers or maceration tubes, of suitable capacity which can be centrifuged. The neck shall be sufficiently large to admit readily the body of the ampul containing the powder. Lightly grease the upper third of the stopper with U. S. P. XI Petrolatum.

2. (*a*) When the digitalis to be assayed is contained in an ampul, the powder shall be weighed as follows: Cut off the ampul head and weigh the body of the ampul and contents, within five minutes, to the nearest milligram. Empty the contents into the above-described container in which previously has been placed 25 cc. of menstruum (U. S. P. XI, p. 397) accurately measured from a burette. Reweigh the empty ampul, thus determining the weight of the digitalis removed. Add sufficient additional menstruum so that the total volume of menstruum corresponds to 10 cc. for each gram of powder taken.

(*b*) When the digitalis to be assayed is packaged in other containers than ampuls, weigh, by difference, from a glass-stoppered weighing bottle to the nearest milligram, about 3 Gm. of the powder and transfer it, as directed in (*a*) to the maceration tube.

3. Shake the mixture of powder and menstruum for 24 ± 2 hours at 25° ± 5° C. by mechanical means (preferably involving end-over-end tumbling), which continuously brings the solid material into fresh contact with the liquid phase.

4. Replace the glass stopper with a rubber stopper and centrifuge at from 1000 to 1500 r. p. m. for at least ten minutes (assuming that the diameter of the centrifuge is not less than 10 inches). Decant the supernatant macerate into a glass-stoppered Pyrex bottle of suitable capacity through a purified cotton plug placed sufficiently loosely in the stem of a 3- to 4-cm. funnel so that the whole process of decantation and filtration can be completed in less than five minutes.

5. Store in a refrigerator at 5° ± 5° C. This preparation should not be used if more than fifteen days old.

(*B*) *Preparation of Test Dilutions.*—Prepare, in a suitable graduated vessel, 10 cc. of test dilution for each of the three dosage levels at which each preparation is to be tested in accordance with the schedule provided[1] in order that each dose, in cc. per Gm. of frog, will be in 0.01 cc. of a liquid containing 20 to 25% by volume of C_2H_5OH. Use the dilutions within six hours after preparation.

(*C*) *The Frogs.*—The frogs shall be *Rana pipiens* (Schreber), weighing between 15 and 50 Gm. In any one assay the lightest frog shall not weigh less than two-thirds of the weight of the heaviest frog. No frogs shall be used which have been previously used for any purpose. (Use only healthy

[1] To conserve space, the schedule of dilutions has been omitted.

and preferably dark-colored, *i. e.*, normally expanded melanophores, male frogs. If it is necessary to use frogs of both sexes or both dark- and light-colored frogs, the respective proportions of such frogs shall be the same in the groups receiving each dose of each preparation.) No females obviously swollen with eggs (provisionally: upon autopsy the weight of the ovaries shall not exceed 10% of the body weight) shall be used.

Prior to use in an assay, the frogs shall be stored in running water below 15° C. (preferably for one week). Six to thirty hours prior to use, transfer to the assay tank somewhat more than 200 frogs meeting the specifications given above. Place the frogs in containers (preferably individual cages for all assays) in which water is maintained at a depth of about 1 cm. and at a temperature of 20° ± 0.5° C. during the assay.

Immediately before injection express the urine from the frog, dry the frog and weigh it to within 0.5 Gm.

Divide the frogs into suitable subgroups and assign at random to each subgroup one of the 12 individual doses. For example, with five frogs in each subgroup and the doses designated as indicated below, the order of dosing the subgroups might be as follows:

g, D, j, A, l, F, i, C, k, B, h, E
h, C, l, D, g, B, j, F, i, E, k, A
j, A, l, C, g, D, i, F

Method	Dosage Level	Sample 1	Sample 2
1-Hr.	Low	A	D
	Intermediate	B	E
	High	C	F
18-Hr.	Low	g	j
	Intermediate	h	k
	High	i	l

(*D*) *Method of Injection into the Lymph Sac.*—Use a tuberculin syringe of ½-cc. capacity, graduated in 0.01 cc. and fitted with a 1¼-inch, 24-gauge needle. The graduations of the syringe should be not less than 3 cm. over all.

Inject through the corner of the mouth into the ventral (abdominal) lymph sac with the end of the needle well below the pectoral girdle avoiding intramuscular injection. If the skin of the frog is accidentally punctured, discard the frog.

(*E*) *Interpretation of Results.*—(1) *One-Hour Method:* (*a*). Pith and open the frogs 58 (±2) minutes after injection and examine them two minutes after opening.

(*b*) Record the condition of the heart at the time of examination indicating the following:

A = Ventricle in systolic standstill, or passes into systolic standstill on gentle mechanical stimulation, while the auricles are widely dilated.

B = Any other stated, but definite, category or categories different from above, which are considered positive.

(*c*) Absorption: Where any question arises as to the completeness of absorption, estimate and record, by checking against observations with known volumes in normal frogs, whether the residual fluid corresponds roughly to more or less than one-fourth of the dose injected.

(2) *Eighteen-Hour Method:* Examine 18 (±2) hours after injection and record the number of dead frogs in each group, considering any frog dead from which a pain reflex cannot be elicited by pinching the toes with forceps or by other suitable means. Examine the heart of each frog considered dead, and record the number of those which are beating.

DISCUSSION OF THE DIRECTIONS

In drafting the above directions the steering committee was helped by the many suggestions made by the collaborators (5). Each detail of the procedure was described in order to minimize the possibility of differences in the interpretation of the directions.

The three-dose design facilitated the application of objective criteria (6) for evaluating the two assay procedures. To equalize the information contributed by each dosage level, only two-thirds as many frogs were to be injected with the intermediate dose as with the low and high doses, respectively. Practical considerations limited the number of frogs in a single unit assay to 200 or less so that most collaborators used 10 or 15 frogs per dosage level.

From preliminary data submitted by one of the collaborators (5) the degree of absorption seemed to be influenced by the volume of the test dilution injected. To favor better absorption in the 1-hour method the volume was limited to 0.01 cc. per Gm. of frog and the same volume was used in the 18-hour procedure in the interest of uniformity within the comparison. In view of the concentrated test dilutions, a 0.5-cc. syringe was specified. In deference to custom, injections were to be made into the abdominal lymph sac caudad to the pectoral girdle, although some available data showed that more complete absorption could be expected in one hour from the smaller pectoral lymph sac immediately cephalad to the pectoral girdle.

From a practical standpoint it was suggested that a ±2 hours' leeway be allowed in reading the reactions in 18-hour assays. In accord with another suggestion (7) the "positive" frogs in the 18-hour assays were to be autopsied to determine whether the hearts were in systolic standstill. All reports were submitted on forms supplied the collaborators, a practice which not only promoted greater uniformity in the extent and detail of the data submitted but also aided greatly in the analysis of the results.

RESULTS OF THE FIRST COMPARISON

The original data of the First Comparison have been reduced to the values given in Table I for each assay. The assays of each laboratory have been numbered chronologically, assay No. "1-2," for example, signifying "Comparison 1, assay No. 2." Although duplicate assays were requested, three collaborators submitted data on only one assay by each method. In Laboratory 1227 the frogs for the 18-hour assays were stored for two weeks prior to use in the assay tank at 20° C. while those for the 1-hour assay were kept at 10 ± 2° C.; moreover, the 1-hour and 18-hour assays were not carried out simultaneously in accord with instructions. Because of these and other differences, the data of this laboratory provide a less valid comparison of the two procedures and only one 18-hour assay could be used. Laboratory 1223 obtained 0 and 100% responses from the low and high levels of the same series of doses in 18-hour assay No. 1-2 and Laboratory 1237 was able to use only two doses, one of which gave 100% response. Their data could not be handled by the method of calculation in the particular form supplied (8) so that no values appear in cols. 4 and 5. However, by extending the method to take into consideration the general character of the other 18-hour data reported by the collaborator a substantial amount of information was salvaged in each of these cases and later where similar data were encountered.

TESTS FOR THE VALIDITY OF INDIVIDUAL ASSAYS

(*A*) *Linearity of Dosage-Effect Curves.*—Before discussing the agreement between the observed potency and the true potency revealed after the assays were completed, it is appropriate to evaluate the individual assay data in the light of what might be expected as a result of normal biological variation. The data of cols. 4, 5, 6, 8 and 9, Table I, obtained from the original data in the course of calculating the potency provide a basis for this evaluation.

Normally, the relationship between the logarithm of the dosage of digitalis and the probit corresponding to the percentage effect produced by its injection (either systolic arrest in one hour or death in eighteen hours) can be plotted as a straight line. Thus the three doses of each sample in each assay provide three points for which the best fitting line may be calculated by the method of "maximum likelihood" (9). If the middle point departs from linearity more than would be expected by chance, this is revealed by the statistical procedure known as the "chi-square (χ^2) test." Since the chi-squares are additive, the values for the two linearity tests in each assay as a whole may be combined. Column 4 in Table I lists the combined χ^2 values with the two "degrees of freedom"[2] for each assay of the First Comparison.

Speaking conservatively, χ^2 values in col. 4 exceeding 4.6 are to be regarded as warning signals on the grounds that the odds are 10:1 against encountering departures from linearity of that magnitude in normal assay data. Ruling out personal or technical errors of dilution, etc., such departures are due presumably to unequal sensitivity among the subgroups of frogs receiving different dosage levels of digitalis. χ^2 values exceeding 4.6 are observed in three of the 40 assays, which is within the frequency to be expected from normal sampling variation. Where such values were encountered, however, the observed rather than the theoretical variance was used in calculating s_M (6), the standard error of the assay. This concession to conservatism yields considerably larger standard errors for these assays so that, as discussed below, they contribute less to the weighted mean. There was no preference between the 1-hour and the 18-hour methods on this score; the χ^2 values for the 1-hour technique totaling 35.06 with 40 degrees of freedom and for the 18-hour method, 30.66 with 37 degrees of freedom, both well within the error of sampling. In general, therefore, the frogs used by the collaborators were satisfactorily "randomized."

(*B*) *Parallelism of Dosage-Effect Curves.*—Within the limits of 0 and 100% effects, the graphical plot of the line relating dosage and effect rises more or less steeply with increasing dose. The slope ("*b*") of this line expresses numerically its steepness or the change in effect measured in probits for each tenfold increase in dosage (as from log dose 0.0 to 1.0). Since the object of a digitalis assay is to determine the magnitude of a difference in potency between the unknown and the reference standard, the most precise assay procedure is that which enables the assayist to distinguish the smallest differences in dose, or the procedure characterized by the steepest slope.

Thus throughout this study the slope of each individual dosage-effect line was of great interest. When two samples are qualitatively alike, as in the present case, the curves relating effect and log-dose in a valid assay should be parallel within the limits of the sampling error. This has been tested by computing χ_b^2 with one degree of freedom separately for each assay as listed in col. 5 of Table I. In only one of 38 cases where the data could be calculated is there a possibly significant departure from parallelism, as would be indicated ordinarily by a χ_b^2 value exceeding 3.8 ($P = 0.05$). Since two χ^2 values as large as this (4.1) might be expected by chance with a series of this length, this particular assay has not been disqualified. From a statistical standpoint, the χ_b^2 values are lower than would be expected, totaling 9.36 for twenty 1-hour assays and 13.20 for the eighteen 18-hour assays. This may arise from the fact that the assays were not designed primarily for the estimation of slope. In assays planned, as these were, to measure potency with the greatest efficiency only large discrepancies in slope appear significant. None of the three 18-hour assays lacking an estimate of χ_b^2 in Table I give indications of a significant departure from parallelism.

The demonstrated agreement in the slopes of the two dosage-effect lines of each assay confirms the basic premise that a pair of parallel lines may represent fairly the six values on standard and sample. Due to the nature of the data it is clear that some uncertainty must exist with respect to the position of these lines. It is equally clear, however, that some one location is more probable than any other for the data in hand. The maximum likelihood calculation serves to reveal this most probable position as well as to measure, in units of dosage of digitalis, the horizontal distance between the lines (*i. e.*, the potency) and the degree of uncertainty associated with that measure (*i. e.*, the standard error of the potency). The characteristic or statistic common to these parallel lines is their relative steepness or slope which is a composite of the individual values for Samples 1 and 2 in each assay. This composite value is given with its standard error in col. 6.

With one exception, the composite slope of the 18-hour assay is greater than that for the 1-hour assay conducted simultaneously on similar frogs. These data demonstrate the principal advantage of the 18-hour over the 1-hour procedure and the one which alone accounts for the greater precision of the former method as indicated by the generally lower standard errors in the individual assays (col. 7) and in the weighted means (cols. 10 and 11). The agreement between the slopes observed in different assays and different laboratories is discussed in a later section.

ESTIMATES OF RELATIVE POTENCY FROM INDIVIDUAL ASSAYS

Probably the data in col. 7 of Table I have the greatest immediate interest since they show the potency of Sample 2 computed separately from the data of each individual assay and expressed as a percentage of that of Sample 1. These potency values are to be compared with 140%, the known, true potency of Sample 2 relative to Sample 1, the latter having been prepared by adding 80 Gm. of inert marc to a 200-Gm. portion of Sample 2. Listed also are the approximate standard errors of the percentage potencies. All values were computed originally in logarithmic units ($M \pm s_M$) (6) but after conversion to percentages have been rounded off to the nearest integer. The two or

[2] "Degrees of freedom" ("*n*") is a statistical term (9, 10) for the number of independent comparisons afforded by the data with respect to a given point. It is needed to determine from tables of χ^2 (10, 11) the probability ("*P*") that the data do not differ from what would be expected with normal sampling variation. Values of *P* of 0.05 or less indicate a "significant" departure from expectation

more individual potency estimates by the same procedure in a given laboratory appear to differ considerably although in no case is the difference significant.

However, three 1-hour and two 18-hour assays (indicated by superscript letter *a* in col. 7) vary from 140% by more than twice their standard errors. Since on the average, only two of the 40 values would be expected to differ so widely, the discrepancy requires some examination. With one exception, other assays which by virtue of larger standard errors are consistent with the known potency differ as much or more than these five values. Thus part of the discrepancy seems to arise from adventitiously small standard errors. The likelihood of this explanation is shown in the next section. The tendency to underestimate the potency will be considered later in discussing the combined results.

TABLE I.—COMPILED DATA OF THE FIRST COMPARISON

Lab. No. (1)	Assay No. (2)	Method (3)	χ^2 (4)	χ_b^2 (5)	Composite Slope (6)	Observed Potency ± s.e. (7)	Weighted Mean Slope 1-Hr. (8)	Weighted Mean Slope 18-Hr. (9)	Weighted Mean Potency 1-Hr. (10)	Weighted Mean Potency 18-Hr. (11)
1221	1-1	1	0.96	0.01	7.5 ± 1.9	129 ± 12	6.3 ± 1.0	8.4 ± 1.5	122 ± 8	132 ± 7
		18	1.87	0.74	13.5 ± 3.6	119 ± 6[a]				
	1-2	1	2.73	0.59	6.7 ± 2.0	106 ± 12[a]				
		18	0.03	0.07	4.3 ± 3.3	164 ± 38				
	1-3	1	0.81	0.20	5.3 ± 1.4	137 ± 19				
		18	0.32	1.91	8.4 ± 1.9	139 ± 12				
1223	1-1	1	0.98	0.53	4.0 ± 1.7	126 ± 24	5.6 ± 1.0	14.7 ± 2.0	133 ± 12	145 ± 5
		18	0.34	0.83	12.7 ± 2.3	148 ± 9				
	1-2	1	0.14	0.76	6.6 ± 1.3	132 ± 15				
		18	....	....	29.0 ± 6.5	160 ± 7[a]				
	1-3	18	0.59	4.14	15.8 ± 4.7	138 ± 6				
1226	1-1	1	1.34	0.78	7.8 ± 3.4	130 ± 12	7.3 ± 2.4	8.5 ± 1.4	137 ± 9	150 ± 11
		18	5.03	0.13	8.9 ± 1.9	135 ± 16				
	1-2	1	4.24	1.13	6.8 ± 3.4	144 ± 22				
		18	2.11	0.00	8.2 ± 1.9	163 ± 16				
1227		18	....	....	.....	.....	5.7 ± 1.4	10.2 ± 3.6	125 ± 15	126 ± 10
		18	2.71	0.01	10.2 ± 3.6	126 ± 12				
		1	3.47	0.91	6.8 ± 2.2	114 ± 19				
		1	7.55	0.11	4.9 ± 1.7	180 ± 54				
1228	1-1	1	1.23	0.61	7.8 ± 1.9	149 ± 14	6.1 ± 1.3	15.9 ± 2.6	149 ± 12	133 ± 4
		18	4.40	2.28	13.6 ± 3.5	137 ± 23				
	1-2	1	0.54	0.08	4.8 ± 1.7	145 ± 19				
		18	1.04	0.44	19.0 ± 4.0	130 ± 5				
1229	1-1	1	1.03	0.20	5.9 ± 1.3	141 ± 18	5.7 ± 0.6	9.2 ± 1.1	126 ± 8	140 ± 6
		18	6.32	0.03	8.3 ± 2.4	161 ± 27				
	1-2	1	1.05	0.70	7.2 ± 1.4	121 ± 13				
		18	0.09	0.05	10.2 ± 2.0	150 ± 11				
	1-3	1	1.95	1.54	4.8 ± 1.2	111 ± 17				
		18	0.76	0.51	10.7 ± 2.4	139 ± 9				
	1-4	1	1.54	0.08	5.3 ± 1.2	135 ± 18				
		18	0.66	0.57	7.3 ± 2.2	127 ± 12				
1235	1-1	1	0.44	0.36	8.2 ± 2.3	144 ± 12	8.2 ± 2.3	18.5 ± 3.3	....	....
		18	0.68	1.02	18.5 ± 3.3	142 ± 6				
1237	1-1	1	3.68	0.21	3.6 ± 2.1	172 ± 46	3.6 ± 2.1	11.8 ± 2.9	....	....
		18	....	....	11.8 ± 2.9	137 ± 12				
1238	1-1	1	0.51	0.17	10.5 ± 2.0	123 ± 8[a]	8.8 ± 1.4	10.1 ± 1.8	119 ± 7	143 ± 7
		18	2.43	0.03	12.3 ± 3.7	142 ± 8				
	1-2	1	0.81	0.27	7.4 ± 1.8	114 ± 11[a]				
		18	0.58	0.35	9.4 ± 2.0	144 ± 12				
1241	1-1	1	0.05	0.09	4.5 ± 3.7	165 ± 25	4.5 ± 3.7	6.7 ± 1.8	...	...
		18	0.05	0.10	6.7 ± 1.8	163 ± 17				

[a] See text regarding these assays.
[b] The report indicates these assays were carried out singly in the order shown on four successive days.

Weighted mean potency, all laboratories—1-hr... 131.3 ± 3.4.
Weighted mean potency, all laboratories—18-hr... 139.2 ± 2.1.

COMBINATION OF RESULTS

(*A*) *Slope.*—The uncertainty or error of even the composite slope from the two curves in these assays is greater than is desirable. Hence, it would be advantageous to combine the data between assays and even between laboratories, if they could be considered as independent estimates of essentially the same slope. By applying the χ^2 test it has been found that the slopes for all assays by each collaborator by each method are indeed mutually consistent; when totaled for all collaborators, $\chi_b^2 = 7.41$ ($n = 10$) for the 1-hour assays and 12.34 ($n = 10$) for the 18-hour assays. Both indicate a very satisfactory agreement with the hypothesis that differences in slope between assays *within* laboratories in this comparison are transitory and of no consequence. Hence the weighted mean slopes in cols. 8 and 9 of Table I have been computed.

Inspection of the combined values for each of the 10 collaborators shows not only a consistently steeper slope for the 18-hour than for the 1-hour method but, equally striking, no apparent correlation between the slopes for the two methods in each laboratory, an impression which may be confirmed by calculation. The study had been so planned that if laboratories differed from one another in slope primarily because some were using frogs of more uniform susceptibility than others, one would expect to find this reflected in both the 1-hour and

the 18-hour assays such that the respective composite slopes would be correlated. Since they are not, the consistency of the slopes *between* laboratories has been tested by computing χ_b^2. None of the 1-hour slopes differ significantly from their weighted mean value of $b_c = 5.954 \pm 0.368$ ($\chi_b^2 = 8.86$, $n = 9$). On the other hand, the 18-hour slopes do differ significantly from their weighted mean value of $b_c = 9.965 \pm 0.928$ ($\chi_b^2 = 24.17$, $n = 9$). This finding must be interpreted as indicating that the 18-hour method does not give the same dosage-effect curve in all laboratories although, as shown in the preceding paragraph, the curves appear to be the same from one assay to the next in any individual laboratory. If this evidence of heterogeneity is neglected temporarily to permit comparison of the weighted mean slope values for the two methods, it is seen that the difference (4.011) is over four times its standard error (± 0.998), showing clearly that the 18-hour method gives a significantly steeper dosage-effect curve.

(*B*) *Potency.*—The considerations leading to looking upon the weighted mean slope values as being characteristic of the assay for each laboratory have an important bearing upon combining the potency results. In a well-planned assay, the difference in the mean effects of standard and unknown is relatively small. Hence the role played by the slope value as a conversion factor for translating differences in effect into dosage units is small enough that random variations in the slope have unimportant consequences. This cannot be said of the role it plays in determining the standard error of the potency estimate. Since the error varies inversely with the slope quite apart from any differences in the mean responses of the frogs, the chance encounters of widely varying slope values are reflected in the calculated standard errors.

It is an established principle that the information contributed by an assay is inversely proportional to the square of its standard error; it is equally well established and logical that in combining a series of assay values into a weighted average, each should be weighted according to the information it supplies. Thus the result of the chance variation in slope seen from assay to assay is that the weights assigned to the individual potency estimates are frequently inordinately low or high. Inasmuch as each collaborator carried out too few assays to effect any stabilization through the sheer weight of large numbers, it seemed possible to make use of the demonstrated fact that the respective mean slopes given in cols. 8 and 9 best characterize all the assays of each laboratory. Hence the data were recomputed (where two or more assays were reported) using the mean slope for the laboratory instead of the respective composite slope.

The recomputed potencies differ little from those given in col. 7, two-thirds of them agreeing within 2 per cent. However, the recomputed standard errors are considerably "stabilized", *i. e.*, the five-fold range of the individually-calculated 1-hour s_M values (± 0.0312 to ± 0.1507) is reduced to a three-fold range (± 0.0352 to ± 0.1125).

The recomputed standard errors of two of the five divergent values in col. 7 exceed one-half the respective discrepancies from the true potency, leaving three still significantly wide of the true value of 140 per cent. While this number might occur in normal sampling, it may be noted that two of them are from Laboratory 1221, one by each method. This coincidence suggests strongly that the extracts used in the first two days' assays differed less in potency than the digitalis from which they were prepared. The third divergent value (Assay 1-2, Laboratory 1238) is supported by its duplicate; as noted below, the weighted average of the two is significantly below the true value. However, assays conducted on the same pair of liquid preparations by the 18-hour method agree very closely with the true potency. This collaborator reported difficulty in preparing the test dilutions which was due, it developed subsequently, to following the directions so meticulously that an inordinately long time was taken to evaporate the excess alcohol. Because the 18-hour doses were small enough not to require much evaporation the difficulty would have affected only the 1-hour results. The significance of this discrepancy was not diminished by the recomputation which, however, did show clearly that the error listed for the 18-hour assay 1-2, Laboratory 1223, is too low because of the chance occurrence of an exceptionally high slope value. Finally, it may be noted that of the results differing considerably from 140 per cent, but not significantly in view of their large standard errors, none assumed significance by virtue of a reduction of the errors through recomputation.

The weighted averages of the recomputed data are given in cols. 10 and 11. Of these, only the 1-hour average of Laboratory 1238 differs significantly from 140 per cent. (Although the rounded-off 1-hour average for Laboratory 1221 appears to be lower by more than twice its average standard error, critical

test shows the difference is not quite significant within the accepted limits of $P = 0.05$.)

All of these laboratory averages and unreplicated values agree with the respective weighted grand averages of 131.3 ± 3.4 for the 1-hour method and 139.2 ± 2.1 for the 18-hour method. The latter result agrees excellently with the known value but the 1-hour value is considerably low; such a divergence would not be expected as the result of normal variation oftener than once in about 25 similar trials. However, without the 1-hour data of Laboratory 1238, about which there is some question on technical grounds, the weighted average is 134.4 ± 3.9 which is clearly not significantly lower than 140 per cent. Considering these facts, therefore, a conclusion that the 1-hour method is definitely less reliable than its 18-hour counterpart is not warranted.

By way of recapitulation it may be said that the objectives of this Comparison were achieved in that the relative merits of the two methods are clearly shown. The dosage-effect relationships of both methods agree with normal biological expectancy and the dosage-effect curve of the 18-hour method is significantly steeper in most laboratories. Neither method appeared to present any exceptional technical difficulties. Both clearly discerned the 40 per cent difference existing between the two samples supplied but agreement of the 18-hour result with the known potency is notably better. This observation is consistent with the smaller observed standard errors of the 18-hour method arising from its steeper dosage-effect curve. From the standpoint of establishing a U. S. P. digitalis reference standard as well as manufacturing U. S. P. Powdered Digitalis, the results show clearly that a blend of a strong powder and inert marc will exhibit its expected potency upon assay.

THE SECOND COMPARISON

General Plan.—The Second Comparison concerned the extraction of digitalis preparatory to assay. The reproducibility of a hot extraction procedure was to be compared with that of the cold extraction (maceration) used in the First Comparison. For this purpose, a single sample of digitalis powder (Sample 3) was distributed to all collaborators, who were requested to prepare at least two extracts by each of the two extraction procedures. To provide a common standard against which these extracts could be compared, tincture (Sample 4) derived from the same specimen of digitalis powder was likewise distributed.[3]

ASSAY DIRECTIONS

The 18-hour method was chosen for this Comparison because of its greater accuracy in the First Comparison. The special directions (12) were based upon preliminary tests by two members of the steering committee which showed that 3-Gm. quantities of digitalis powder could be extracted with hot alcohol more readily in a "rubber extractor"[4] than in the usual Soxhlet apparatus. Because 95% alcohol instead of U. S. P. menstruum (76% alcohol) was used in both the hot and cold extraction techniques, an appropriate dilution schedule was provided. Since a longer period was allowed for the digitalis action, the dosage volume of test dilution was increased to 0.02 cc. per Gm. of frog. Otherwise the directions were the same as in the First Comparison.

RESULTS OF THE SECOND COMPARISON

The Second Comparison was carried out in April and May, 1940; the ten reports submitted include data on two or more pairs of assays. The results are summarized in Table II, of which cols. 1 and 2 are similar to those in Table I. Column 3 indicates the method of extraction and whether the extract is the first, second or third made by that method; hence "H-3" designates the third fresh extract prepared by the hot extraction procedure. The potency values in col. 4 have been calculated for each assay as in the First Comparison. Although both extracts were compared with a common standard, the potency of each has been computed independently of the other as a percentage of Sample 4. This neglects a certain amount of available information on the slope of the dosage-effect curve prevailing at the time of the assay but gives the truest picture of the variation to be expected between single assays by this method.

Two "failures" are indicated in Table II. The first is in the data of Laboratory 1226 which reported kills of 8/15, 9/10 and 8/15 for Extract H-1. As indicated by the χ^2 test these data are quite beyond the limits of normal sampling variation with respect to both linearity and slope. Because of this evidence of heterogeneity no attempt was made to get a potency estimate from the data. The second instance in which no potency estimate was made from the data reported is Assay 2-1 of Laboratory 1227. Here kills of 14/15, 10/10 and 15/15 were reported for the three doses chosen of Extract

[3] The powder for this study was composited and the tincture was prepared as follows: After assay (13), all the drug samples were reduced to a uniform degree of fineness in a ball mill and then uniformly blended in the same mill, practically all exposure to air and loss of material being avoided. The composite sample thus prepared was of a fineness of approximately No. 60 powder, and the moisture content was less than 8%. The total amount of blended drug weighed about 100 pounds. Separate samples of each lot were reserved. The mixed drug, stored in a large, airtight drum, later became known as the "Study Powder."

For the preparation of the tincture, 500 Gm. of the composite powder were placed with 5000 cc. of alcohol (U. S. P.) in a 2-gallon bottle which was fastened in a pill coating pan, as nearly upright as possible and rotated continuously for twenty-four hours. After settling, the clear supernatant liquid was decanted and bottled for distribution.

[4] Described in most chemical supply catalogues as "Extraction Apparatus for Rubber Analysis."

M-1. The choice of the latter represented an underestimation of the potency so that with the chance encounter of an unusually high mortality, very little information was obtained as to the exact potency.

The composite slopes for each assay and for all the assays by each laboratory are given with their standard errors in cols. 5 and 6, respectively.

Table II.—Compiled Data of the Second Comparison

Lab. No. (1)	Assay No. (2)	Extract No. (3)	Potency, % (4)	Composite Slope for Assay (5)	Composite Slope for Laboratory (6)	χ_b^2 (7)	Weighted Mean Potency Maceration (8)	Weighted Mean Potency Hot Extraction (9)
1222	2-1	M-1	109 ± 6	11.3 ± 2.1	.....	.....	.....	.....
		H-1	110 ± 8					
1223	2-1	M-1	100 ± 4	16.6 ± 2.4	12.4 ± 1.5	4.96(.08)	101 ± 4	[a]
		H-1	70 ± 3					
	2-2	M-2	103 ± 7	9.2 ± 2.4				
		H-2	91 ± 9					
	2-3	H-3	119 ± 9	11.2 ± 3.2				
1226	2-1	M-1	98 ± 12	6.9 ± 2.8	7.5 ± 1.7	0.08(.37)	101 ± 9	.....
		H-1	.....					
	2-2	M-2	103 ± 15	7.9 ± 2.2				
		H-2	112 ± 12					
1227	2-1	M-1	.....	6.3 ± 3.6	10.1 ± 1.1	2.42(.50)	102 ± 5	119 ± 5
		H-1	124 ± 33					
	2-2	M-1	102 ± 8	10.4 ± 2.1				
		H-1	117 ± 9					
	2-3	M-2	113 ± 11	12.1 ± 2.0				
		H-2	128 ± 8					
	2-4	M-2	98 ± 7	9.0 ± 2.0				
		H-2	116 ± 11					
1228	2-1	M-1	101 ± 7	12.4 ± 3.2	13.6 ± 1.5	0.30(.95)	102 ± 3	118 ± 3
		H-1	126 ± 11					
	2-2	M-1	108 ± 7	14.4 ± 3.0				
		H-1	125 ± 5					
	2-3	M-2	100 ± 4	14.3 ± 3.0				
		H-2	112 ± 7					
	2-4	M-2	103 ± 6	13.0 ± 2.9				
		H-3	111 ± 4					
1229	2-1	M-1	104 ± 6	14.4 ± 2.6	11.1 ± 1.1	9.60(.02)	104 ± 4	108 ± 4
		H-1	101 ± 6					
	2-2	M-2	121 ± 28	9.1 ± 2.2				
		H-2	104 ± 8					
	2-3	M-2	114 ± 12	7.7 ± 2.0				
		H-2	128 ± 15					
	2-4	M-3	97 ± 5	16.3 ± 2.5				
		H-3	111 ± 5					
1231	2-1	M-1	84 ± 12	6.7 ± 1.9	6.6 ± 0.9	2.79(.42)	99 ± 6	104 ± 6
		H-1	102 ± 8					
	2-2	M-1	105 ± 10	8.8 ± 1.8				
		H-1	110 ± 8					
	2-3	M-2	121 ± 31	4.6 ± 1.8				
		H-2	119 ± 28					
	2-4	M-2	96 ± 9	6.2 ± 1.7				
		H-2	94 ± 12					
1235	2-1	M-1	100 ± 6	9.8 ± 2.9	10.1 ± 2.1	0.02(.88)	102 ± 5	105 ± 5
		H-1	105 ± 9					
	2-2	M-2	102 ± 5	10.4 ± 3.0				
		H-2	102 ± 9					
1237	2-1	M-1	114 ± 9	7.7 ± 2.2	5.5 ± 1.2	1.37(.50)	117 ± 10	122 ± 10
		H-1	111 ± 11					
	2-2	M-2	109 ± 18	4.6 ± 2.1				
		H-2	98 ± 28					
	2-3	M-3	124 ± 17	4.7 ± 1.9				
		H-3	126 ± 18					
1238	2-1	M-1	103 ± 5	12.7 ± 2.9	14.2 ± 2.1	0.47(.50)	102 ± 4	121 ± 4
		H-1	121 ± 6					
	2-2	M-2	99 ± 6	15.6 ± 3.0				
		H-2	120 ± 6					

[a] The Hot Extraction potency values for this laboratory are obviously too heterogeneous to combine in a weighted mean and, as suggested by the collaborator, indicate progressively more complete extraction inasmuch as fresh extracts were employed in each assay.

Weighted mean potency, all laboratories—Maceration procedure . . . 102.8 ± 1.4.
Weighted mean potency, all laboratories (except 1223)—Hot Extraction procedure . . . 114.1 ± 1.7.

That the composite assay slope values are satisfactorily consistent with their respective means is shown by the χ^2 values and the corresponding probabilities (in parentheses) in col. 7. In only two instances, Laboratories 1222 and 1229, is the probability notably low (0.10) that the several assay composites agree, within sampling error, with the mean slopes. In the rest of the series the data are consistent with the conclusion reached in the First Comparison that the composite slope for each laboratory best represents the dosage-effect relationship characteristic of that laboratory's assays. Hence the weighted mean potency values for each laboratory (cols. 8 and 9) have been computed using the laboratory composite slope value.

POTENCY ESTIMATES

(*A*) *Maceration.*—The cold maceration extracts prepared individually by each collaborator were expected to show essentially the same potency as that of the standard tincture supplied. That this expectation is borne out in striking fashion is seen in the data of cols. 4 and 8, Table II. In no case is an individual assay value significantly different from 100%, and all the laboratory weighted means of the assays of the cold extracts fall between 99 and 104%, with the single exception of Laboratory 1237. The latter found the cold extract consider-

ably but not significantly stronger than the standard tincture; there is no apparent explanation of this singular discrepancy.

The weighted mean of the entire series is 102.8 ± 1.4%, indicating clearly that the maceration process is capable of yielding highly un form extracts not only within a given laboratory but from one laboratory to another. Although the 2.8% excess potency in the laboratory-prepared extracts is just significant by conventioval standards, it is unimportant practically. Thus it appears that the process yields extracts of uniform potency with both small and relatively large quantities. It would be interesting to ascertain whether the process is applicable to manufacturing lots of digitalis.

(*B*) *Hot Extraction.*—The assay data for the hot extracts present a different situation. The risk of incomplete extraction of the activity can scarcely be eliminated in a procedure of this kind. However, only one collaborator (Laboratory 1223) encountered significant ($P = 0.015$) discrepancies in potency between three different hot extracts, in the first of which incomplete extraction is certainly indicated. As noted in Table II, Laboratories 1228 and 1229 also prepared three separate hot extracts; the first extract of the former was almost significantly ($P = 0.07$) stronger than the second and third.

The data of Laboratory 1223 have not been included in striking a weighted average of the hot extraction data because of the doubt as to complete extraction. The weighted average of 114.1 ± 1.7% shows clearly that the hot extract prepared as directed is more potent than the cold extract from the same specimen of digitalis. This observation, taken with the fact that the full volume of 1.0 cc. per 0.1 Gm. of powder is obtained, indicates that a substantial amount of digitalis activity is lost in the solid matter which is centrifuged off and discarded in the cold extraction procedure. This observation also has important implications from a manufacturer's viewpoint.

In evaluating the results of the Second Comparison, it should be kept in mind that the hot extraction technique was relatively unfamiliar to the collaborators. However, in view of the highly uniform results with the simpler cold maceration procedure, it is clearly the method of choice for obtaining a standard assay preparation from a reference digitalis powder.

THE THIRD COMPARISON

General Plan.—The Third Comparison of the study consisted of assays of the composited Study Powder against International Standard Digitalis (1936) by both the 1-hour and 18-hour methods. The objective was (*a*) to supplement the information gained in the First Comparison on the relative merits of the two methods and (*b*) to establish the potency of the Study Powder in terms of the currently available international standard.

ASSAY DIRECTIONS

While the plan of the Third Comparison was the same as that of the First Comparison, the directions for the latter were changed because of the decision to inject a volume of 0.02 cc. per Gm. of frog (instead of 0.01 cc.) into the pectoral lymph sac (instead of the abdominal lymph sac); a dilution schedule appropriate for the increased injection volume was included. Each collaborator was supplied with a sample of digitalis powder labeled "Sample No. 5" (which was the same as Sample 3 of the Second Comparison) and two ampuls of the International Standard Digitalis (1936). Other details, including preparing extracts for assay by maceration with U. S. P. digitalis menstruum, were the same as for the First Comparison.

Results of the Third Comparison. The data, compiled in Table III, were received from 11 collaborators and with one exception each collaborator submitted results on at least two assays by each of the two methods. The assays were carried out in August, September and October of 1940. In form, Table III is the same as Table I. Two "failures" were reported for the 18-hour method, both by the same collaborator, who observed an unexpectedly high mortality from administration of the international standard.

The observations of the First Comparison are confirmed although, as discussed later, the superiority of the 18-hour procedure with respect to steepness of slope is not so marked in these data. As in the First Comparison, Laboratory 1238 reported the highest weighted mean slope observed in the 1-hour series; in fact, the value 13.4 reported by this collaborator is significantly greater than those reported by several others. In common with some others, this collaborator observed no significant difference between the two methods with respect to slope.

The potency estimates for each assay, calculated as in the First Comparison, are listed in col. 7 as International Digitalis Units per gram of Sample 5. By both methods, the two or more results of each laboratory are quite consistent not only one with another but also with the respective laboratory weighted means (cols. 10 and 11). In contrast to the good agreement between replicates *within* laboratories, the poor agreement *between* laboratories is disappointing. In the 1-hour data, the weighted average of 11.2 ± 0.05 for Laboratory 1222 is significantly lower than that of 14.7 ± 0.10 reported by Laboratory 1224. This discrepancy in laboratory weighted averages is reflected further by the χ^2 test since $\chi_M^2 = 17.5$ with $n = 10$ and $P = 0.06$. Taken alone, this is not evidence of significant variation from normal sampling although such a discrepancy would be expected only once in 16 trials in assays of identical extracts.

In the 18-hour series the variation between laboratories is much greater although, as noted above, the variation within each laboratory is negligible. Thus $\chi_M^2 = 32.2$ with $n = 9$, indicating that such poor agreement between laboratory averages would be expected less than once in a thousand times in assays of extracts identical in potency. Since it is scarcely credible that the samples of digitalis supplied the collaborators were not of the same potency, the discrepancy must be attributed to variations in preparing the extracts.

This observation is in contrast to the close agreement obtained in the Second Comparison by a procedure which differed apparently only in minor details, such as using a menstruum of 95% instead of 76% alcohol. If it is possible that this favored a more uniform potency in the individual liquid extracts prepared by each collaborator, the point should be checked. In addition, the abdominal lymph sac was used in the Second Comparison while the pectoral lymph sac was used in these assays. It may be that the vagaries of the frog have been overcome sufficiently by the restrictions on the technique and design of the assay that other sources of error begin to emerge.

This hypothesis is supported by the generally good agreement between the 1-hour and 18-hour results. While two collaborators report a consider-

ably higher value by the 1-hour method, only in the case of Laboratory 1231 does the χ^2 test indicate ($P = 0.04$) that the difference between methods is beyond the realm of normal sampling variation.

The respective weighted averages of the 1-hour and 18-hour series, obtained in the usual manner, are 12.17 ± 0.22 and 11.47 ± 0.18 International Digitalis Units per gram. Because of the marked heterogeneity of the data it represents, the 18-hour weighted average cannot be regarded as reliable within the usual limits of twice the calculated standard error. Consequently, the 6% difference between the two averages, is insignificant. Finally, the doubt thrown on the validity of the 18-hour average by the variation between laboratories is dispelled considerably by the fact that the laboratory means are distributed equally above and below the weighted average of the whole series.

Except for the variation from laboratory to laboratory discussed above, the conclusions reached in this Comparison confirm those of the First Comparison. The substantial agreement in the potency estimates by the two methods and the steeper slope of the 18-hour method were observed in both Comparisons. Upon conclusion of this Comparison, therefore, the steering committee recommended the adoption of some form of the 18-hour method in U. S. P. XII whereupon it was decided to base the dilution of the Study Powder to the desired potency of 10 International Digitalis Units per gram on the 18-hour weighted average.

TABLE III.—COMPILED DATA OF THE THIRD COMPARISON

Lab. No. (1)	Assay No. (2)	Method (3)	χ^2 (4)	χ_h^2 (5)	Composite Slope (6)	Observed Potency ± s. e. (7)	Weighted Mean Slope 1-Hr. (8)	Weighted Mean Slope 18-Hr. (9)	Weighted Mean Potency 1-Hr. (10)	Weighted Mean Potency 18-Hr. (11)
1222	3-1	1	0.83	0.76	9.9 ± 3.5	11.4 ± 0.09				
		18	2.10	0.28	10.9 ± 2.8	10.8 ± 0.07				
	3-2	1	0.84	0.01	12.9 ± 3.6	11.0 ± 0.06	11.4 ± 2.5	12.6 ± 1.7	11.2 ± 0.05	10.9 ± 0.05
		18	5.28	0.46	13.6 ± 2.0	11.3 ± 0.06				
1223	3-1	1	2.55	0.12	7.7 ± 2.3	10.6 ± 0.09				
		18	1.22	0.16	8.6 ± 2.4	10.7 ± 0.08				
	3-2	1	1.34	0.10	7.7 ± 2.3	11.5 ± 0.10				
		18	0.17	0.11	10.9 ± 2.8	10.7 ± 0.07	6.5 ± 0.8	10.9 ± 1.4	11.8 ± 0.07	10.6 ± 0.04
	3-3	1	0.44	0.62	6.4 ± 1.1	12.8 ± 0.16				
		18	0.93	0.04	12.3 ± 2.2	10.2 ± 0.07				
	3-4	1	1.05	1.11	5.4 ± 1.7	12.4 ± 0.17				
1224	3-1	1	4.22	0.52	7.0 ± 1.9	15.4 ± 0.24				
		18[a]	.....	.....	.....	.....				
	3-2	1	0.86	0.00	8.6 ± 2.4	14.0 ± 0.11	7.6 ± 1.5	11.0 ± 2.3[b]	14.7 ± 0.10	.....
		18[a]	.....	.....	.....	.....				
1226	3-1	1	4.42	0.11	6.9 ± 2.8	15.0 ± 0.26				
		18	2.33	0.17	17.1 ± 3.2	12.1 ± 0.05				
	3-2	1	8.45	2.14	7.4 ± 2.3	12.1 ± 0.23	7.2 ± 1.8	11.4 ± 2.1	13.9 ± 0.16	12.6 ± 0.06
		18	0.74	1.59	6.9 ± 2.8	14.1 ± 0.17				
1227	3-1	1	0.44	0.01	4.8 ± 1.6	10.4 ± 0.16				
		18	2.09	2.30	5.4 ± 2.3	12.6 ± 0.16				
	3-2	1	1.05	0.14	6.4 ± 1.5	12.1 ± 0.13	5.6 ± 1.1	5.7 ± 1.6	11.3 ± 0.10	12.3 ± 0.13
		18	7.56	1.66	6.0 ± 2.3	11.5 ± 0.26				
1228	3-1	1	0.05	0.70	6.0 ± 1.9	11.5 ± 0.13				
		18	1.01	2.94	7.4 ± 2.0	10.2 ± 0.11				
	3-2	1	1.10	0.40	10.2 ± 2.0	12.3 ± 0.08				
		18	3.73	1.00	7.7 ± 1.8	13.6 ± 0.12	7.1 ± 1.1	7.9 ± 1.1	11.3 ± 0.06	12.0 ± 0.06
	3-3	1	3.76	0.10	5.7 ± 1.7	10.6 ± 0.17				
		18	0.66	0.32	8.5 ± 1.9	11.8 ± 0.09				
1229	3-1	1	0.37	0.93	5.4 ± 1.4	13.0 ± 0.17				
		18	2.21	0.26	8.4 ± 2.4	12.4 ± 0.10				
	3-2	1	2.52	0.67	6.4 ± 1.5	11.4 ± 0.13				
		18	1.60	0.16	5.3 ± 2.4	11.9 ± 0.15	5.2 ± 0.8	6.9 ± 1.4	12.6 ± 0.10	11.6 ± 0.07
	3-3	1	1.46	0.13	3.9 ± 1.4	14.2 ± 0.25				
		18	0.85	1.30	7.0 ± 2.3	10.6 ± 0.11				
1231	3-1	1	1.03	4.26	4.2 ± 1.8	11.7 ± 0.20				
		18	0.17	4.64	10.0 ± 2.5	11.0 ± 0.08				
	3-2	1	1.78	2.04	4.9 ± 1.7	12.8 ± 0.18				
		18	0.92	0.43	9.7 ± 2.7	10.4 ± 0.07	5.8 ± 0.8	10.6 ± 1.6	13.3 ± 0.08	10.6 ± 0.04
	3-3	1	4.48	0.05	7.5 ± 1.6	14.1 ± 0.22				
		18	0.86	2.77	12.8 ± 3.2	10.4 ± 0.06				
	3-4	1	0.66	0.00	6.1 ± 1.5	14.0 ± 0.16				
1235	3-1	1	0.54	0.08	6.2 ± 2.5	13.4 ± 0.15				
		18	4.81	0.00	11.8 ± 2.1	11.7 ± 0.06	6.7 ± 1.7	11.8 ± 2.1	12.1 ± 0.09	11.2 ± 0.08
	3-2	1	0.67	0.30	7.2 ± 2.3	10.7 ± 0.11				
1237	3-1	1	3.50	0.00	6.8 ± 1.7	12.3 ± 0.19				
		18	0.53	0.92	10.5 ± 2.4	10.4 ± 0.07				
	3-2	1	2.34	0.26	10.1 ± 2.3	10.5 ± 0.09	7.9 ± 1.4	8.9 ± 1.8	11.6 ± 0.08	10.4 ± 0.06
		18	0.03	0.90	7.3 ± 2.5	10.5 ± 0.10				
1238	3-1	1	0.04	0.08	11.5 ± 3.4	12.7 ± 0.07				
		18	2.16	1.47	17.0 ± 3.8	12.9 ± 0.06				
	3-2	1	0.05	0.09	15.7 ± 3.6	12.6 ± 0.06	13.4 ± 2.5	13.6 ± 2.6	12.7 ± 0.04	13.3 ± 0.05
		18	1.31	0.21	10.7 ± 3.5	13.9 ± 0.10				

[a] 100% kills resulted from both the intermediate and high doses of International Standard in both of these assays.
[b] This composite slope value represents data of Sample 5 only.

Weighted mean potency, all laboratories— 1-hr. method . . . 12.17 ± 0.022 International Digitalis Units.
Weighted mean potency, all laboratories—18-hr. method . . . 11.47 ± 0.018 International Digitalis Units.

THE FOURTH COMPARISON

General Plan and Directions.—At first, a portion of the Study Powder was diluted to the desired potency of 10 International Digitalis Units per gram by adding 23.35[5] Gm. of exhausted marc to 150 Gm. of the

[5] The quantity, 23.35 Gm., was based on the value of 11.55 International Digitalis Units, which is the weighted average of the 18-hour values given in col. 7, Table III. The recomputation, which gives the value of 11.47 as the weighted average of all the 18-hour data, was not carried out until this report was compiled.

original Study Powder (represented by Samples 3 and 5 of the Second and Third Comparisons, respectively). The resulting powder was distributed for a confirmatory assay principally among the members of the steering committee (14). The assays were conducted by the 18-hour procedure. For assay, the provisionally diluted powder was extracted by maceration using a ratio of 0.1 Gm. of powder for each cc. of U. S. P. digitalis menstruum. Similar extracts were prepared from the International Standard Digitalis (1936) using, however, only 0.08 Gm. per cc. of menstruum (Laboratory 1221 used a ratio of 0.1 Gm. per cc.).

TABLE IV.—COMPILED DATA OF THE FOURTH COMPARISON

Lab. No. (1)	χ^2 (2)	Slope (3)	Potency—International Units per Gm. ± s. e. Individual Values (4)	Potency—International Units per Gm. ± s. e. Weighted Mean (5)
1221	1.47	5.7 ± 1.8	10.6 ± 1.3	10.6 ± 1.3
1222	3.22	10.2 ± 2.4	9.5 ± 0.7	
	0.14	7.5 ± 3.4	10.6 ± 1.2	
	5.62[a]	13.9 ± 3.5	8.4 ± 0.5	9.4 ± 0.3
	6.86[a]	16.3 ± 3.2	9.1 ± 0.4	
	1.93[a]	7.0 ± 2.2	9.1 ± 0.9	
1227	1.05	7.2 ± 2.4	8.8 ± 0.9	
	0.37	11.3 ± 2.5	10.4 ± 0.7	
	6.51[b]	6.2 ± 2.3	8.9 ± 1.8	9.9 ± 0.5
	9.74[b]	11.1 ± 2.6	10.0 ± 1.5	
1229	1.50	11.6 ± 2.5	9.7 ± 0.7	
	2.39	10.1 ± 2.9	10.8 ± 0.8	
	1.18	16.7 ± 4.5	10.3 ± 0.5	10.2 ± 0.3
	3.96[a]	7.7 ± 2.3	9.7 ± 0.7	
1231	2.94	9.5 ± 2.4	10.9 ± 1.1	
	5.76[b]	12.3 ± 2.5	9.2 ± 0.8	
	1.83	7.3 ± 2.3	9.1 ± 0.9	9.8 ± 0.4
	1.44	12.2 ± 4.0	10.4 ± 0.6	
	4.26	7.2 ± 2.3	8.4 ± 1.3	

[a] Four-dose assay; therefore $n = 4$. [b] χ^2 exceeds 4.6 so that P is less than 0.10.

Weighted mean potency, all assays . . . 9.8 ± 0.2 International Units per Gm.

THE RESULTS OF THE FOURTH COMPARISON

The assays were conducted in February, 1941. The results (Table IV) calculated as previously, resemble those of earlier comparisons making a detailed discussion unnecessary.

The χ^2 values in col. 2, Table IV, have been calculated exactly as those given in col. 4, Table I. As noted, several 4-dose assays were reported in this Comparison and in all of these the χ^2 value is satisfactorily low, since with 4 degrees of freedom it would have to exceed 7.8 to assume significance at the conservative level of $P = 0.10$. However, in 3 of the 3-dose assays χ^2 exceeds 4.6, making the probability less than 0.10 (since $n = 2$) that the dosage-effect relationship is linear. Allowance has been made for this heterogeneity, so that these results, identified by [b] in col. 2, contribute less to the weighted average of the laboratory and of the whole series. This finding confirms again the necessity for providing an internal check on the consistency of the results with those expected under normal sampling conditions. Also it emphasizes the very real necessity for insuring the greatest possible equivalence of the frogs in the subgroups at the various dosage levels. The weighted averages of the five collaborators agree very well ($\chi_M^2 = 3.8$ with $n = 4$) with the average potency of 9.79 International Digitalis Units per gram of the provisionally diluted powder. While the observed potency is not significantly below that desired, it nevertheless appears that slightly too much inert marc was added.

THE FIFTH COMPARISON

General Plan.—The purpose of the Fifth Comparison was threefold: (*a*) to determine the potency, in International Digitalis Units, of the main stock of the Study Powder now diluted on the basis of the earlier results, (*b*) to furnish data on the uniformity of standard tinctures prepared from reference digitalis powders and (*c*) to test a two-dose assay design which was under consideration for U. S. P. XII. The first two points checked questions treated in earlier phases of the study; the two-dose assay design represented a compromise between the one-dose, standard curve method (15) and the three-dose design used in the first four Comparisons. At least three doses are required for both a determination of the slope of the dosage-mortality curve and a check on the linearity or "goodness of fit" of the observations to straight lines. Analysis of the data of the first three Comparisons, given in detail below, shows that while the slope of the 18-hour data appeared to vary not only from one laboratory to another but also within the confines of a single laboratory from time to time (thereby ruling out the general use of a standard curve), it appeared constant within a given assay. Hence the calculation of potency may be based on the pair of parallel lines best fitting the two sets of points without com-

puting the best-fitting lines for standard and unknown separately. It is therefore possible to retain the desired internal checks in a simpler design calling for only two doses each of the reference standard and the preparation being assayed.

The material distributed (labeled "U. S. P. Digitalis Powder for Fifth Comparison Study, May, 1941") represented digitalis powder prepared by adding 2.005 Kg. of exhausted marc to 15.200 Kg. of Study Powder. This material represented a slight upward adjustment of the provisional dilution assayed in the Fourth Comparison. The International Standard Digitalis (1936) was used as the reference standard and liquid extracts were prepared by the maceration technique.

ASSAY DIRECTIONS

Detailed directions for conducting the assay (16) and for calculating the results (17) were supplied. Briefly, the directions called for preparing two separate liquid preparations from the study sample and the international standard. The assays were to be planned, facilities permitting, so that the uniformity of potency of two extracts prepared from the same powder could be checked. The technical details were:

"(*A*) *Standard Preparations of Digitalis.*—Weigh to the nearest milligram approximately 3 Gm. of standard digitalis powder and transfer it with a minimum of exposure to atmospheric moisture to a dry, hard glass, glass-stoppered container of at least 50 cc. capacity and designed suitably for centrifuging. Add with shaking sufficient menstruum consisting of four parts of alcohol, by volume, and one part of water, by volume, so that the total volume of menstruum added corresponds to 10.0 cc. for each Gm. of Study Powder taken and 10.0 cc. for each 0.8 Gm. of International Standard Digitalis taken. Stopper the container with a glass stopper the upper third of which is greased lightly with petrolatum, U. S. P. Shake the mixtures of powder and menstruum for 24 ± 2 hours at 25 ± 5° C. by a mechanical means which continuously brings the solid material into fresh contact with the liquid phase. Centrifuge the mixtures sufficiently to permit the supernatant tinctures to be decanted into dry, glass-stoppered bottles of suitable capacity. Preserve under refrigeration.

"(*B*) *The Frogs.*—For the purposes of this assay, select frogs of the variety *Rana pipiens* Schreber weighing between 15 and 50 Gm., free from gross evidence of disease or injury and among which are no females obviously swollen with eggs. Store the frogs prior to the assay, if necessary, in running water at a temperature below 15° C. For any one assay, select frogs sufficiently uniform in size so that the weight of the lightest frog is not less than two-thirds of that of the heaviest. Six to thirty hours prior to the assay, distribute sufficient frogs in the assay tank in such a way that the groups to receive each of the doses of the two standard tinctures being assayed, and the frogs to serve as controls, will be as nearly alike as practicable to obtain with respect to weight, sex, and exposure to environmental influences.

"(*C*) *Selection of the Dosage of Digitalis.*—In order to conserve time, assume provisionally that the proper doses of both tinctures are 2.82 cc./Kg. of frog (log dose 0.45) and 3.76 cc./Kg. of frog (log dose 0.575). Prepare test dilutions of one standard preparation of each powder corresponding to these doses and inject each into 10 frogs. If, as is to be expected from the results of the Third and Fourth Comparisons, the kills are substantially the same from the corresponding doses of the two standard powders, combine them to make available the information on 20 frogs on each of the two dosage levels. This will reveal the absolute sensitivity to digitalis of the frogs in use and indicate whether the dosage interval provisionally suggested above should be altered in any way. On the basis of this information, select doses such that (*a*) the low doses will be expected to kill at least one of a group of fifteen frogs injected and the high doses will kill no more than fourteen of a group of fifteen frogs injected; and (*b*) the ratio of the high dose to the low dose is the same for both tinctures and does not exceed 1.5 but is sufficiently great to ensure that the respective mortalities produced in groups of 15 frogs by the two doses will not differ by less than three frogs.

"(*D*). *Preparation of the Test Dilutions.*—Prepare the test dilutions according to the table below taken from earlier directions (17).

Log Dose	Dose/ Kg. of Frog, Cc.	Vol. of Assay Prepn./ 10 Cc. of Test Dilution, Cc.	Make to the desired volume with special diluent containing the volume indicated below of U.S.P. XI Digitalis menstruum *q. s.* 100 cc. with distilled water
0.30	2.00	1.00	21.88
0.35	2.24	1.12	20.84
0.40	2.51	1.26	19.64
0.45	2.82	1.41	18.26
0.50	3.16	1.58	16.64
0.55	3.55	1.78	14.75
0.60	3.98	1.99	12.51
0.65	4.47	2.23	9.86
0.70	5.01	2.51	6.67
0.75	5.62	2.81	2.82
			Remove by evaporation[a]
0.80	6.31	3.16	4% of weight of sample
0.85	7.08	3.54	12% of weight of sample
0.90	7.94	3.97	18% of weight of sample
0.95	8.91	4.46	24% of weight of sample
1.00	10.00	5.00	29% of weight of sample
1.05	11.22	5.61	34% of weight of sample
1.10	12.59	6.30	38% of weight of sample
1.15	14.13	7.06	42% of weight of sample
1.20	15.85	7.92	45% of weight of sample

[a] Carry out this evaporation as follows: Place the required amount of digitalis preparation (containing 74–75% alcohol) in a weighed 50-cc. beaker and determine the weight of the sample. Arrange for a stream of clean, dry air from a nozzle consisting of a length of glass tubing (6 mm. inside diameter) protruding about a millimeter from the center of the large end of a cork, the diameter of which is 6–10 mm. less than that of the 50-cc. beaker being used. (This type of nozzle reduces to insignificance the amount of room air brought into contact with the evaporating liquid and thereby eliminates the only important source of variation, due to the humidity of the room air, so far discovered in this method of preparing test dilutions of equal alcoholic concentrations.) With the end of the nozzle 1–2 cm. below the rim of the beaker, evaporate to the desired level, in terms of original weight of the sample, and transfer to a suitable graduated vessel and make up to volume with distilled water.

"(*E*). *Injection of the Test Dilutions.*—To determine the volume of the appropriate test dilution to be injected, remove each frog from the assay tank immediately before injection, dry it and weigh to the nearest 0.5 Gm. after expressing the urine by gentle pressure upon the urostile. Multiply the weight in Gm. by 0.02 and inject the volume in cc., indicated by the product. Use a tuberculin syringe of suitable capacity and design fitted with a 1½ inch, 24-gauge needle. Inject through the corner of the mouth into the pectoral lymph sac, directing

the needle toward the opposite foreleg in such a manner as to avoid puncturing the pectoral girdle. Return the injected frog to the assay tank, discarding any frog in which the skin is accidentally punctured.

"(*F*). *Interpretation of the Results.*—At the end of a period 18 ± 3 hours after the injection, examine the frogs and record the number of dead in each group injected with each test dilution. Consider any frog dead in which the righting and pain reflexes are abolished."

CALCULATION OF THE RESULTS OF THE FIFTH COMPARISON (18)

Ascertain, by the tests given below, whether the data are adequate to determine the potency of the preparation being assayed and, if so, calculate the potency observed and the extent to which it is indicated the true potency of the preparation may vary from that observed.

Definitions:

The terms used in describing the method of calculation are defined as follows:

Standard = Standard Preparation of Digitalis.

Unknown = Preparation being assayed.

$(X_1)_S$ and $(X_1)_U$ = the logarithms of the low doses of Standard and Unknown, respectively, such dosage being expressed as cc. of preparation per Kg. of frog.

x = the logarithm of the ratio of the high dose (Dose 2) to the low dose (Dose 1) for both Standard and Unknown.

Y_1 and Y_2 = the probits corresponding to the mortalities produced by Doses 1 and 2, respectively, of both Standard and Unknown.

w_1 and w_2 = the weights proportional to the information supplied by Y_1 and Y_2 relative to the potency of the Unknown.

W_S and W_U = the composite weights associated with $(Y_2 - Y_1)_S$ and $(Y_2 - Y_1)_U$, the differences in the probits corresponding to the mortalities produced by Doses 2 and 1, respectively, of both Standard and Unknown.

Chi-square (χ^2) = a numerical expression denoting whether the difference $Y_2 - Y_1$ for the Standard is consistent with the corresponding difference for the Unknown within the limits of normal sampling variation.

s_e = logarithm of the proportionate increase in dose which, under the conditions of the assay, increases mortality by one probit, *i. e.*, as from 50% to 84%.

$\bar{x}_S$ and $\bar{x}_U$ = the weighted means of the logarithms of Doses 1 and 2 of Standard and Unknown, respectively.

$\bar{y}_S$ and $\bar{y}_U$ = the weighted mean probits for Standard and Unknown, respectively.

M = the logarithm of the observed potency of the Unknown in U. S. P. Digitalis Units per cc.

s_M = the standard error of M.

"Fiducial Limits" = the limits below and above the observed potency which, as here defined, would be presumed to enclose the true potency of the Unknown in 19 out of 20 similar trials.

Transformation of the Original Data:

1. Determine from a logarithm table the value of x.

2. Determine from Table V the corresponding probit, Y, the weight, w, and the product, wY, for each of the observed mortality ratios. Tabulate as in the numerical example given below.

Determination of the Composite Weights:

Compute the composite weight values for the Standard and Unknown by the following equations:

$$W_S = \frac{(w_1 w_2)_S}{(w_1 + w_2)_S} \text{ and } W_U = \frac{(w_1 w_2)_U}{(w_1 + w_2)_U}$$

The Chi-Square Test for Validity:

1. Apply the chi-square test to determine the validity of the assay data by solving the following equation:

Chi-square (χ^2) =

$$\frac{W_S W_U \{(Y_2 - Y_1)_S - (Y_2 - Y_1)_U\}^2}{W_S + W_U}$$

2. If chi-square exceeds 2.71, conclude that either the Standard or the Unknown differed in their mode of action or the groups of frogs were not sufficiently alike at the two dosage levels and repeat the assay.

3. If chi-square is less than 2.71, conclude that the data are valid within the limits of normal sampling variation and proceed with the calculation.

Determination of the Average Sensitivity to a Difference in Dosage:

1. Compute s_e by the following equation:

$$s_e = \frac{x(W_S + W_U)}{W_S(Y_2 - Y_1) + W_U(Y_2 - Y_1)_U}$$

2. If the value of s_e exceeds 0.200 conclude that the frogs employed were too insensitive to a difference in digitalis dosage to be suitable for assay purposes and that the assay must be repeated.

3. If the value of s_e is less than 0.200, proceed with the calculation.

Determination of the Observed Potency:

1. Substitute the appropriate values in the following equations to obtain the numerical value of M:

$$\bar{x}_S = \frac{x(w_2)_S}{(w_1 + w_2)_S} + (X_1)_S \text{ and } \bar{x}_U = \frac{x(w_2)_U}{(w_1 + w_2)_U} + (X_1)_U;$$

$$\bar{y}_S = \frac{(w_1 Y_1 + w_2 Y_2)_S}{(w_1 + w_2)_S} \text{ and } \bar{y}_U = \frac{(w_1 Y_1 + w_2 Y_2)_U}{(w_1 + w_2)_U}$$

$$M = \bar{x}_S - \bar{x}_U - s_e(\bar{y}_S - \bar{y}_U)$$

2. Ascertain from a table of antilogarithms the antilogarithm of M which is the numerical value of the observed potency of the Unknown expressed in U. S. P. Digitalis Units per cc.

Determination of the Standard Error:

Compute the standard error of M by the following equation:

$$s_M = \pm s_e \sqrt{\frac{(w_1 + w_2)_S + (w_1 + w_2)_U}{(w_1 + w_2)_S (w_1 + w_2)_U}}$$

Determination of the Fiducial Limits of the Observed Potency:

1. Compute the lower and upper fiducial limits of the observed potency as:

Lower Limit = $(1 - 4.51\, s_M)$ (antilog M) and
Upper Limit = $(1 + 4.51\, s_M)$ (antilog M)

If the lower fiducial limit is greater than 0.80 U. S. P. Digitalis Unit and the upper fiducial limit is less than 1.20 U. S. P. Digitalis Units, consider that the Unknown meets the prescribed requirements.

2. If the observed potency is approximately 1.0 U. S. P. Digitalis Unit but the fiducial limits fall outside the range of 1.00 ± 0.20, consider that the odds are greater than one in twenty that the Unknown may not meet the prescribed requirements. In this case repeat the assay and combine the new results with earlier results by the means given below until the fiducial limits fall within the prescribed requirements of 1.00 ± 0.20 U. S. P. Digitalis Units per cc.

3. If the observed potency is not approximately 1.0 U. S. P. Digitalis Unit per cc., adjust the Unknown and repeat the assay.

Determination of the Weighted Mean of Two or More Assays:

1. If more than one assay meeting the above requirements is completed, combine the results by solving the following equations:

$$\overline{M} = \frac{\left(\frac{1}{s_M}\right)_1^2 M_1 + \left(\frac{1}{s_M}\right)_2^2 M_2 \ldots + \left(\frac{1}{s_M}\right)_n^2 M_n}{\left(\frac{1}{s_M}\right)_1^2 + \left(\frac{1}{s_M}\right)_2^2 \ldots + \left(\frac{1}{s_M}\right)_n^2}$$

where $\overline{M}$ is the logarithm of the weighted mean of the two or more (up to n) values of M obtained in separate assays and designated above by appropriate sub-scripts.

2. Determine the standard error of $\overline{M}$, $s_{\overline{M}}$, by the following equation:

$$s_{\overline{M}} = \pm \frac{1}{\sqrt{\left(\frac{1}{s_M}\right)_1^2 + \left(\frac{1}{s_M}\right)_2^2 \ldots + \left(\frac{1}{s_M}\right)_n^2}}$$

3. Determine the fiducial limits indicated by the combined results of the two or more assays by substituting $\overline{M}$ for M and $s_{\overline{M}}$ for s_M in the equations given above for the lower and upper fiducial limits.

Discussion of the Directions.—The calculation[6] given above is a modification of the previous solution (5) developed especially for the two-dose assay of digitalis in the light of the experience gained in the first four Comparisons of this study. To provide objective means for identifying "poor" assays, two internal checks were offered in the calculation. One, the chi-square test, indicates whether the dosage-mortality curves for standard and sample are parallel within the limits of normal sampling variation. The proposed limiting value of chi-square, 2.71, corresponds to odds of 1 in 10, this level being chosen, on conservative grounds in preference to the usual 1 in 20 probability. The second measures the average sensitivity of the frogs to a difference in digitalis dosage and reflects the steepness of the observed dosage-mortality curve. Inasmuch as the composite slopes for most collaborators approximated 9.0 for the 18-hour assay in the first four Comparisons, the value of $s_e = 0.20$ (corresponding to $b_e = 5.0$) was taken arbitrarily as indicating that the frogs were too insensitive to an increase in digitalis dosage to justify basing an estimate of potency upon their reactions.

RESULTS OF THE FIFTH COMPARISON

Nine collaborators, all of whom had participated earlier in the study, submitted data on the Fifth Comparison. All of their assays were completed in July and August, 1941. Both the basic data and the calculated results are presented in Table VI.

Columns 1 and 2 of Table VI are self-explanatory and indicate that each collaborator carried out assays on two different days and one collaborator extended his observations over four days. Column 3 indicates the extract number identified as sug-

NUMERICAL EXAMPLE ILLUSTRATING THE TWO-DOSE CALCULATION

TABULATIONS AND CALCULATIONS

Prepn.	BASIC DATA Dosage, Cc./Kg.	Log Dose	Observed Mortality		From Table V Y	w	wY
Standard							
Dose 1	2.51	0.40	4/15		4.38	8.3	36.35
Dose 2	3.55	0.55	11/15		5.62	8.3	46.65
				Sums		16.6	83.00
				Diff.	1.24		
Unknown							
Dose 1	2.82	0.45	2/15		3.89	6.0	23.34
Dose 2	3.98	0.60	10/15		5.43	8.9	48.33
				Sums		14.9	71.67
				Diff.	1.54		

$W_S = \frac{(8.3)(8.3)}{16.6} = 4.20$

$\bar{x}_S = \frac{0.15(8.3)}{16.6} + 0.40 = 0.475$

$\bar{y}_S = \frac{83.00}{16.6} = 5.00$

$W_U = \frac{(6.0)(8.9)}{14.9} = 3.58$

$\bar{x}_U = \frac{0.15(8.9)}{14.9} + 0.45 = 0.54$

$\bar{y}_U = \frac{71.67}{14.9} = 4.82$

$\chi^2 = \frac{(4.2)(3.58)(1.24 - 1.54)^2}{4.20 + 3.58} = 0.17$ (<2.7) so that data on Standard and Unknown are consistent

$s_e = \frac{0.15(4.20 + 3.58)}{4.20(1.24) + 3.58(1.54)} = 0.109$ (<0.2) so that frogs were sufficiently sensitive to differences in dosage

$M = 0.475 - 0.540 - 0.109(5.00 - 4.82) = -0.0746$; Antilog $M = 0.84$ U. S. P. Digitalis Units per cc.

$s_M = 0.109\sqrt{\frac{16.6 + 14.9}{(16.6)(14.9)}} = \pm 0.037$; Standard Error = $\pm 2.3(0.037)(0.84) = \pm 0.07$ U. S. P. Digitalis Units per cc.

Fiducial Limits = $\{1 \pm 4.51(0.037)\}0.84 = 0.70$ and 0.98 U. S. P. Digitalis Units per cc.

CONCLUDE: The potency of the product must be increased and the assay repeated since there is considerable chance that the true potency is below 0.80 U. S. P. Digitalis Unit per cc., the minimum allowed.

[6] The principles and formulas for calculating the two-dose assay were worked out by C. I. Bliss and put in their present form by the author. The derivation of the method is to be published separately by Dr. Bliss.

gested in the directions, *i. e.*, "U-1" refers to the first extract of Study Sample 5, whereas "S-2" refers to International Digitalis Standard extract No. 2, etc. Columns 4 and 6 give the logarithm of the low and high doses, repectively, the dosage being in cc. of the standard extract per 10 Kg. of frog. Columns 5 and 7 indicate, respectively, the number of dead frogs observed at the end of the assay period in the groups of 15 receiving the low and high doses. In cols. 8, 9 and 10 are given the values of x, χ^2 and s_e, each of which is defined in the directions for the two-dose assay calculations. Column 11 lists the logarithm (and its standard error) of the potency value calculated from the basic data. The potency (and its standard error) of Study Sample 5 is given in International Digitalis Units per gram in col. 12. Column 13 gives the weighted averages of the potency values in col. 12.

It is remarkable that of the 55 assays given in Table VI (from which are omitted only the few "preliminary assays" included in the reports) only three failed because no frogs survived in one or both of the groups receiving the high dosage. While a potency estimate is possible by a special treatment of these data, none has been made. Apparently assuming that a different dosage-response relationship would hold for the "standard" than for the "unknown," two collaborators chose unequal dosage intervals for the two preparations (see col. 8), so that appropriate modification of the calculation procedure had to be made in these instances.

Seven of the nine collaborators reported assays which failed to meet one or both of the criteria set up in the directions. Of the 55 assays, seven show a χ^2 exceeding 2.71 (col. 9), a proportion agreeing not unfavorably with the 1 in 10 expectancy. Of the seven high values, four exceed 3.84, the "5 per cent level," or a 1 in 20 expectancy; on the grounds that the latter indicates definite heterogeneity the results have been omitted from the weighted averages.

In nine instances s_e exceeds the arbitrary limit of 0.200 although in only two cases is the value high enough to invalidate any estimate of potency. One of these values, 0.352 (Assay 1, Laboratory 1224), indicates a composite slope of only 2.84, which is much lower than the characteristic slope for the 18-hour assay. The other value, −0.882 (Assay 1, Laboratory 1235), presents a situation not anticipated in the directions, *i. e.*, that of a *negative* composite slope; although less than 0.200, it clearly indicates that the frogs did not react to digitalis in the characteristic manner.

TABLE V.—GIVING THE PROBIT, Y, THE WEIGHT, w, AND THE PRODUCT, wY, CORRESPONDING TO ALL MORTALITY RATIOS FROM 1/15 TO 14/15 FOR GROUPS OF 15 ANIMALS

Mortality Ratio	Probit, Y	Weight, w	Product, wY
1/15	3.50	4.0	14.000
2/15	3.89	6.0	23.340
3/15	4.16	7.4	30.784
4/15	4.38	8.3	36.354
5/15	4.57	8.9	40.673
6/15	4.75	9.3	44.175
7/15	4.92	9.5	46.740
8/15	5.08	9.5	48.260
9/15	5.25	9.3	48.825
10/15	5.43	8.9	48.327
11/15	5.62	8.3	46.646
12/15	5.84	7.4	43.216
13/15	6.11	6.0	36.660
14/15	6.50	4.0	26.000

It will be noted that Laboratory 1223 observed a very low potency in both comparisons of the first extract of the International Standard ("S-1") with that of the study sample ("U-1"). Inspection of the data of cols. 4–7, Table VI, reveals clearly that "S-1" was much stronger than "S-2." Since the discrepancy between these two assay results and those of all the rest of the series represents a variation that is quite beyond the realm of normal sampling error, the values have been omitted from the weighted average. The number of assays which fail to meet the two criteria is not excessive and accounts for only six of the 11 assays not included, the remaining five being omitted by virtue of what appear to be errors in technique and judgment. Thus 44 assays, or four-fifths of those reported, are included in the weighted average.

As noted above, only two laboratories (1229 and 1231) encountered difficulty in meeting the requirement that there should be at least one death and one survivor from every low and high dose, respectively. This indicates that the lack of flexibility of the two-dose design, as compared with those having three or more doses of each preparation, is no particular disadvantage, although with more than two doses a considerably greater amount of information can be salvaged in instances of no deaths or no survivors from one of the doses.

In general, the values of s_e are larger than would be expected from the earlier data, which are characterized by a composite s_e of 0.11 (the reciprocal of the over-all composite slope of 9.2). In the Fifth Comparison data, s_e averages 0.136 ± 0.006 for the 44 assays included in the weighted average. This value is significantly greater than 0.11, so that a significantly lower slope value obtains for this series. The variation in slope between assays and between collaborators is about the same as in the earlier Comparisons and it appears difficult to reconcile the low slope values so generally obtained in this Comparison with the earlier data.

The precision of the two-dose procedure is indicated by the standard errors listed in cols. 11 and 12, Table VI. The average s_M value (col. 11) is ±0.0479 which corresponds to a potency variation of ±11%. Hence, on the average, the observed potency of a single two-dose assay would not deviate from the true potency by ±20% oftener than once in twenty times. Other factors being equal, those laboratories characterized by a high slope would expect deviations of this magnitude less frequently, while the less fortunate in this respect should expect them oftener. Thus it appears that the proposed two-dose assay design is quite practicable, a conclusion that represents achievement of one of the three objectives of this Comparison.

A second objective was to determine the uniformity of extracts prepared from the same standard powder by the same technique. Except for the singular discrepancy noted in Laboratory 1223 there is so little evidence of heterogeneity between the two or more extracts of the same powder that the matter was not put to statistical test. Thus these data confirm those of the Second Comparison and leave unexplained the marked heterogeneity observed in the Third Comparison.

The final objective of the Fifth Comparison is met in the weighted mean potency of these data, which indicates that the Study Sample represented 9.71 ± 0.14 International Digitalis Units per gram. This value is just significantly lower than the expected 10 International Units per gram, an observation which is discussed further below.

TABLE VI.—COMPILED BASIC AND CALCULATED DATA ON THE FIFTH COMPARISON

Lab. No. (1)	Day No. (2)	Ext. No. (3)	Basic Data: Low Dose Log (4)	Low Dose Kill (5)	High Dose Log (6)	High Dose Kill (7)	Calculated Data: x (8)	x^2 (9)	s_x (10)	Potency: Log ± s. e. (11)	Potency: Int. Dig. Units ± s. e. (12)	Weighted Mean (13)
1223	1	U-1	0.795	8	0.845	13	0.05	2.58	0.117	0.7805 ± 0.0831	(6.0 ± 1.2)[a]	
		S-1	0.574	11	0.624	10						
		U-2	0.845	8	0.895	13	0.05	0.24	0.042	0.9945 ± 0.0155	9.9 ± 0.4	9.7 ± 0.3
		S-2	0.845	8	0.895	14						
	2	U-1	0.754	4	0.829	6	0.075	0.53	0.122	0.7413 ± 0.0409	(5.5 ± 0.5)[a]	
		S-1	0.549	5	0.624	10						
		U-2	0.800	7	0.875	10	0.075	0.25	0.113	0.9357 ± 0.0390	8.6 ± 0.8	
		S-2	0.790	9	0.865	13						
1224	1	U-1	0.625	4	0.750	9	0.125	0.10	0.128	1.0126 ± 0.0437	10.3 ± 1.1	
		S-1	0.625	3	0.750	9						
		U-2	0.625	4	0.750	5	0.125	0.23	0.352	0.8750 ± 0.1179	(7.5 ± 2.0)[b]	
		S-2	0.625	5	0.750	8						
	2	U-2	0.625	1	0.750	8	0.125	1.41	0.122	0.9156 ± 0.0439	8.2 ± 0.8	9.4 ± 0.4
		S-1	0.625	6	0.750	10						
		U-1	0.625	3	0.750	8	0.125	0.04	0.127	0.9632 ± 0.0434	9.2 ± 0.9	
		S-1	0.625	4	0.750	10						
		U-2	0.625	3	0.750	10	0.125	0.25	0.114	1.0222 ± 0.0397	10.5 ± 1.0	
		S-2	0.625	3	0.750	8						
		U-1	0.625	4	0.750	8	0.125	0.06	0.159	0.9274 ± 0.0535	8.5 ± 1.0	
		S-2	0.625	6	0.750	11						
1226	1	U-1	0.475	4	0.625	9	0.15	0.10	0.154	1.0154 ± 0.0525	10.4 ± 1.2	
		S-1	0.475	3	0.625	9						
		U-2	0.475	5	0.625	6	0.15	3.40	0.210	1.0395 ± 0.0754	11.0 ± 1.9	
		S-2	0.475	1	0.625	8						
	2	U-2	0.475	1	0.625	8	0.15	0.48	0.118	0.9970 ± 0.0440	9.9 ± 1.0	10.4 ± 0.5
		S-1	0.475	2	0.625	7						
		U-1	0.50	1	0.65	5	0.15	0.52	0.201	0.9964 ± 0.0771	9.9 ± 1.8	
		S-1	0.50	2	0.65	4						
		U-2	0.50	2	0.65	9	0.15	2.08	0.188	0.9798 ± 0.0651	9.8 ± 1.5	
		S-2	0.50	5	0.65	7						
		U-1	0.50	4	0.65	13	0.15	0.40	0.101	1.0446 ± 0.0367	11.1 ± 0.9	
		S-2	0.50	3	0.65	10						
1227	1	U-1	0.50	3	0.625	8	0.125	0.05	0.149	1.0211 ± 0.0512	10.5 ± 1.2	
		S-1	0.50	3	0.625	7						
		U-2	0.50	2	0.625	6	0.125	0.89	0.251	0.9822 ± 0.0882	9.6 ± 2.0	
		S-2	0.50	4	0.625	5						
	2	U-2	0.50	3	0.625	9	0.125	0.76	0.161	0.9628 ± 0.0542	9.2 ± 1.2	9.2 ± 0.5
		S-1	0.50	6	0.625	9						
		U-1	0.50	6	0.65	9	0.15	0.31	0.221	0.9603 ± 0.0734	9.1 ± 1.5	
		S-1	0.50	6	0.65	11						
		U-2	0.50	3	0.65	7	0.15	0.02	0.185	0.9144 ± 0.0519	8.2 ± 1.0	
		S-2	0.50	5	0.65	10						
		U-1	0.50	2	0.65	6	0.15	0.10	0.153	0.9382 ± 0.0541	8.7 ± 1.1	
		S-2	0.50	3	0.65	9						
1228	1	U-1	0.525	3	0.675	8	0.15	0.31	0.207	0.9977 ± 0.0704	10.0 ± 1.6	
		S-1	0.525	4	0.675	7						
		U-2	0.525	3	0.675	9	0.15	0.14	0.123	1.0139 ± 0.0436	10.3 ± 1.0	
		S-2	0.525	2	0.675	9						
	2	U-1	0.55	5	0.70	13	0.15	0.01	0.100	1.0448 ± 0.0362	11.1 ± 0.9	
		S-1	0.55	3	0.70	11						
		U-2	0.55	2	0.70	9	0.15	0.24	0.097	0.9338 ± 0.0358	8.6 ± 0.7	
		S-2	0.55	4	0.70	13						
		U-1	0.55	4	0.70	14	0.15	2.61	0.111	1.0118 ± 0.0414	10.3 ± 1.0	
		S-2	0.55	6	0.70	11						
	3	U-1	0.55	3	0.675	12	0.125	2.81	0.121	0.9778 ± 0.0423	9.5 ± 0.9	9.8 ± 0.3
		S-1	0.55	7	0.675	10						
		U-2	0.55	2	0.675	12	0.125	1.01	0.081	0.9460 ± 0.0301	8.8 ± 0.6	
		S-2	0.55	7	0.675	13						
		U-1	0.55	5	0.675	9	0.125	0.08	0.162	1.0148 ± 0.0540	10.4 ± 1.3	
		S-2	0.55	4	0.675	9						
	4	U-3	0.60	9	0.70	11	0.10	2.16	0.123	1.0329 ± 0.0429	10.8 ± 1.1	
		S-3	0.60	5	0.70	12						
		U-4	0.60	5	0.70	9	0.10	0.74	0.104	0.9704 ± 0.0356	9.3 ± 0.8	
		S-4	0.60	5	0.70	12						
		U-4	0.60	8	0.70	12	0.10	0.01	0.137	0.9744 ± 0.0466	9.4 ± 1.0	
		S-3	0.60	7	0.70	11						
1229	1	U-1	0.575	3	0.725	3	0.15	5.37	0.179	0.8807 ± 0.0663	(7.6 ± 1.2)[c]	
		S-1	0.575	2	0.725	11						
		U-2	0.575	4	0.725	8	0.15	1.40	0.140	1.0016 ± 0.0490	10.0 ± 1.1	
		S-2	0.575	2	0.725	10						
	2	U-2	0.575	3	0.725	7	0.15	0.36	0.156	1.0026 ± 0.0549	10.1 ± 1.3	9.6 ± 0.6
		S-1	0.575	2	0.725	8						
		U-1	0.60	4	0.75	13	0.15	..	...	.....	.....	
		S-1	0.60	8	0.75	15						
		U-2	0.60	3	0.75	11	0.15	0.72	0.131	0.9615 ± 0.0454	9.2 ± 1.0	
		S-2	0.60	6	0.75	11						
		U-1	0.60	2	0.75	10	0.15	0.45	0.102	0.9770 ± 0.0557	9.5 ± 1.2	
		S-2	0.60	4	0.75	11						
1231	1	U-1	0.600	8	0.725	8	0.125	5.39	0.193	0.9623 ± 0.0689	(9.2 ± 1.5)[c]	
		S-1	0.625	6	0.750	14						
		U-2	0.600	4	0.725	14	0.125	..	...	.....	.....	
		S-2	0.625	9	0.750	15						
	2	U-2	0.600	5	0.725	14	0.125	3.19	0.111	0.9657 ± 0.0426	9.2 ± 0.9	9.8 ± 0.5
		S-1	0.625	11	0.750	13						
		U-1	0.575	8	0.700	11	0.125	0.31	0.173	0.9980 ± 0.0586	10.0 ± 1.3	
		S-1	0.575	7	0.700	12						
		U-2	0.575	6	0.700	12	0.125	1.73	0.084	0.9990 ± 0.0314	10.0 ± 0.7	
		S-2	0.575	4	0.700	14						
		U-1	0.575	8	0.700	15	0.125	..	...	.....	.....	
		S-2	0.575	2	0.700	15						

TABLE VI.—COMPILED BASIC AND CALCULATED DATA ON THE FIFTH COMPARISON—(*Continued*)

Lab. No. (1)	Day No. (2)	Ext. No. (3)	Basic Data: Low Dose Log (4)	Low Dose Kill (5)	High Dose Log (6)	High Dose Kill (7)	x (8)	χ^2 (9)	s_c (10)	Calculated Data: Potency: Int. Dig. Units Log ± s. e. (11)	Int. Dig. Units ± s. e. (12)	Weighted Mean (13)
1235		U-1	0.65	12	0.725	9	0.10	6.75	0.333	[b, c]	.....	
		S-1	0.65	8	0.725	14						
	1	U-2	0.65	8	0.75	11	0.10	1.86	0.106	0.9770 ± 0.0383	9.5 ± 0.8	
		S-2	0.65	7	0.75	14						
		U-2	0.65	9	0.75	9	0.10					
		S-1	0.65	12	0.725	11	0.075	0.10—0.882		[c]	.....	9.5 ± 0.5
		U-1	0.60	3	0.75	10	0.15					
		S-1	0.55	5	0.65	4	0.10	4.44	0.240	[c]	.....	
	2	U-2	0.60	4	0.75	12	0.15	0.07	0.101	1.0192 ± 0.0359	10.4 ± 0.9	
		S-2	0.60	3	0.725	10	0.125					
		U-1	0.60	4	0.75	8	0.15	1.40	0.129	0.9138 ± 0.0453	8.2 ± 0.9	
		S-2	0.60	5	0.725	13	0.125					
1237		U-1	0.475	4	0.600	8	0.125	0.27	0.143	1.0223 ± 0.0484	10.5 ± 1.2	
	1	S-1	0.55	5	0.675	11						
		U-2	0.50	4	0.625	12	0.125	0.27	0.098	0.9553 ± 0.0346	9.0 ± 0.7	
		S-2	0.475	6	0.600	12						10.0 ± 0.5
		U-1	0.475	5	0.625	11	0.15	0.00	0.131	1.0626 ± 0.0447	11.6 ± 1.2	
	2	S-1	0.55	5	0.675	11	0.125					
		U-2	0.50	6	0.625	13	0.125	0.50	0.115	0.9961 ± 0.0402	9.9 ± 0.9	
		S-2	0.45	5	0.575	10						

Weighted mean of series . . . 9.71 ± 0.14.

[a] These values excluded from means. See text.
[b] This value excluded from means because of high s_c value.
[c] These values excluded from means because of high χ^2 value.

TABLE VII.—SUMMARIZED COMPARISON OF THE 1-HR. AND 18-HR. METHODS

Lab. No. (1)	Source of Frogs (2)	1-Hr. Method: Average ED_{50}, Cc./Kg. 1st (3)	3rd (4)	Composite Slope 1st (5)	3rd (6)	18-Hr. Method: Average LD_{50}, Cc./Kg. 1st (7)	3rd (8)	Composite Slope 1st (9)	2nd (10)	3rd (11)	All Comparisons (12)	χ^2 [b] (13)	Ratio of Slope, 18-Hr.: 1-Hr. 1st (14)	3rd (15)
1221	Wis.	0.275 (3)[a]	....	6.3	..	0.305	....	8.4	..	..	..	.....	1.33	..
1222	Vt.	....	0.473	...	11.4	....	0.327	....	11.3	12.6	12.1	0.26 (1)	....	1.11
1223	Vt.	0.622	0.594 (4)	5.6	6.5	0.427 (1)	0.350 (3)	14.0	12.4	10.9	12.0	1.54	2.50	1.68
1224	Ill.	....	0.439	..	7.6	....	....	....	....	11.0	....	.....	....	1.45
1226	Wis.	0.493	0.555	7.3	7.2	0.344	0.437	8.3	7.6	11.4	8.8	2.08	1.14	1.58
1227	Wis.	0.374	0.531	5.6	5.6	0.338 (1)	0.398	8.0	10.1	5.7	8.6	4.84	1.43	1.02
1228	Que.	0.360	0.397 (3)	6.1	7.1	0.278	0.389 (3)	15.9	13.6	7.9	10.4	14.07	2.61	1.11
1229	Wis.	0.537 (4)	0.423 (3)	5.7	5.2	0.337 (4)	0.387 (3)	9.2	11.1	6.9	9.3	5.68	1.61	1.33
1231	Wis.	....	0.456 (4)	...	5.8	....	0.422 (3)	....	6.6	10.6	7.6	4.80 (1)	....	1.83
1235	Mich.	0.537 (1)	0.527	8.2	6.7	0.387 (1)	0.421 (1)	18.5	10.1	11.8	12.2	4.83	2.26	1.76
1237	Wis.	0.457 (1)	0.380	3.6	7.9	....	0.344	....	5.5	8.9	6.5	6.21	....	1.13
1238	Vt.	0.624	0.631	8.8	13.4	0.294	0.436	10.1	14.2	13.6	12.2	2.57	1.15	1.02
1241	N. J.	0.270 (1)	....	4.5	....	0.338 (1)	....	6.4	....	....	....	....	1.42	....
Total number of assays		20	28	...	....	17	23	....	....	....	....	....	....	....
Weighted averages		....	....	5.9	6.4	....	....	9.6	9.4	9.1	....	....	1.72	1.37

[a] Values in parentheses indicate the number of assays (cols. 3, 4, 7 and 8) or degrees of freedom (col. 13) if other than two.

CRITICAL COMPARISON OF THE 1-HOUR AND 18-HOUR METHODS

(*A*) *The Observed ED_{50} and LD_{50}.*—In cols. 3, 4, 7 and 8 of Table VII are listed the average amounts of digitalis required for 50 per cent effects in the 1-hour and 18-hour methods as reported in the First and Third Comparisons. In calculating the values for the First Comparison it has been assumed that, after allowance is made for the known difference in potency, the data on Samples 1 and 2 represent two separate estimates of the dose producing, in 50 per cent of the frogs injected, systolic standstill in the 1-hour method ("ED_{50}") and death in the 18-hour method ("LD_{50}"). The values listed, being the antilogs of the arithmetic means of two or more logarithms, are actually geometric means and are expressed in the original units of cc. of tincture per Kg. of body weight of frog. Since the digitalis samples used in these two Comparisons were not the same, the respective ED_{50} and LD_{50} values are not comparable in amount.

(*1*) *Variation in the ED_{50}.*: Examination of the ED_{50} values of the individual assays shows there is relatively little variation from one assay to another. For example, the individual values of Laboratory 1229 in the First Comparison are 0.540, 0.522, 0.514 and 0.510 cc. per Kg. of the tinctures prepared from Samples 1 and 2. The four individual values reported by Laboratory 1231 in the Third Comparison are 0.478, 0.460, 0.482 and 0.408 cc. per Kg. of the tincture prepared from Sample 5. In contrast, there is a great variation between laboratories, as may be judged from the values listed in cols. 3 and 4, Table VII. The two extreme values in col. 3 (Laboratories 1241 and 1238) reflect the discrepancies from the true potency of Sample 2

which are noted in the reports from these laboratories. Nevertheless it is clear that to achieve systolic standstill in comparable proportions of injected frogs some collaborators were forced to inject twice as much digitalis as others.

The variation in ED_{50} is less in the data of the Third Comparison but nevertheless is indicative of a real difference between observers. The difference is brought out well in the data of Laboratories 1222 and 1238, both of which used male frogs purchased from the same dealer; while the unusually high slopes reported by these collaborators (col. 6) indicate that the frogs they used were quite similar, the respective ED_{50} values are significently different ($P = 0.05$). The variation in the ED_{50} in the First Comparison is about twice that observed in the Third Comparison, and it seems probable that this difference is related to the character of the frogs used. The First Comparison was carried out in January and February, when the available frogs had been stored, in hibernation, since the previous fall. The Third Comparison, conducted in the following August and September, doubtless was on freshly caught frogs.

Another possible explanation of the difference noted is that in the First Comparison the directions called for injecting a volume of 0.01 cc. per Gm. into the abdominal lymph sac while the Third Comparison called for injecting 0.02 cc. per Gm. into the pectoral lymph sac. Evidence (19) developed in the period intervening between the two Comparisons indicated that absorption from the pectoral lymph sac is more rapid and less digitalis is required for comparable effects at the end of one hour than if the injection is made into the larger abdominal lymph sac. The collaborators were specifically requested to note any unabsorbed fluid in the lymph sac, and many collaborators reported that absorption was obviously incomplete in one hour. The request to measure roughly the remaining fluid proved impracticably time-consuming so that few data were collected on the point.

Each collaborator was requested to supply information on the end point used in his 1-hour assays. The responses indicate the existence of room for variation in the ED_{50} by virtue of differences of opinion as to what is a "positive reaction." While most collaborators indicated that they demanded absolute systolic ventricular standstill and diastolic auricular standstill in frogs counted "positive," others allowed some irregular auricular contraction and one collaborator stated that the reaction was considered positive when the auricles were in a "long diastolic state and the ventricle was contracted for a long duration after a heart beat." It happened that the latter collaborator was among those reporting a low average ED_{50}. It should follow that any level of effect, consistently applied, should give a valid comparison between the standard and unknown. However, when the active constituents of the drug are as complex as those of digitalis, the risk of assay discrepancies between laboratories will probably increase if appreciably different amounts are used.

(*2*) *Variation in the* LD_{50}: As with the ED_{50}, the variation in the LD_{50} within laboratories is negligible. The variation between laboratories in the LD_{50}, while appreciable, proves to be less than half as great by actual calculation as that seen in the ED_{50}.

As may be noted from Table VII, the two laboratories which reported the lowest ED_{50} by the 1-hour method found a higher LD_{50} by the 18-hour method, and those laboratories reporting the highest ED_{50} found the LD_{50} was only 50 to 70 per cent as large. These observations do not lend themselves to objective evaluation, but they support the conclusion that probably because of its sharper end point the 18-hour procedure leads to greater uniformity between laboratories.

Mention of the sharper end point of the 18-hour method prompts a discussion of the responses to two other questions included in the reports, *i. e.*, the number of deaths observed at the end of the observation period in a similar but uninjected group of frogs and the result of autopsy on all frogs considered dead, whether injected or not. While some collaborators failed to report fully on these points, the data supplied show a remarkable correlation between the incidence of frankly beating hearts in "dead" injected frogs and the incidence of death in the control uninjected frogs. The latter amounted to about 10 per cent of the frogs in some cases. In computing the potency no correction has been made for these two factors, which tend to counteract each other if the control deaths are disregarded and the criterion of a positive reaction is taken as a gross appearance of death coupled with failure to respond to sensory stimulation.

(*B*) *The Slopes of the Dosage-Effect Curves.*—It is notable that the two laboratories which observed the best agreement between the two methods in the First Comparison reported only one assay by each method. These data, however, afford a

very good example of the influence of the slope of the dosage-effect relationship on the precision of the assay. From the standpoint of design, the assays of Laboratory 1241 are practically ideal. The doses selected are equally spaced and so chosen that each of the four low doses produced either 3 or 4 positives of the groups of 15 injected, the intermediate doses gave 4 or 5 positives in the groups of 10 injected, while all four of the high doses produced 11 positives in the respective groups of 15 frogs injected. In contrast, the data of Laboratory 1235 indicate unequal spacing of the doses (in the 1-hour method the log-dose interval is 0.05 between the low and intermediate doses and 0.10 between the intermediate and high doses for both samples and in the 18-hour method the corresponding intervals are 0.05 and 0.10 for Sample 1 and 0.05 and 0.05 for Sample 2). The grouping of the frogs differed from that specified in the directions (one intermediate dose being injected into 15 frogs and only 10 each being used on one low and one high dose). The doses chosen did not result in the best efficiency since 100 per cent kills resulted from both of the high doses used in the 18-hour method, so that they contributed relatively little information. Nevertheless the data of Laboratory 1235 yield calculated standard errors (col. 9, Table I) which were only 8 and 4 per cent of the potency values observed by the 1-hour and 18-hour methods, respectively. Expressed similarly, the corresponding standard errors in the data of Laboratory 1241 are 15 and 10 per cent.

Since by definition the precision of an assay is inversely proportional to the standard error, the 1-hour assay of Laboratory 1241 is only one-half as precise as the 1-hour assay of Laboratory 1235 and only one-fourth as precise as the latter's 18-hour assay. These conclusions stand in the face of the fact that Laboratory 1241 met all the specified requirements and therefore achieved the maximum efficiency in the use of the frogs available. This paradox arises because the frogs used in the assays of Laboratory 1235 were sufficiently alike in their susceptibility to digitalis poisoning (as indicated by the relatively high slopes in both the 1-hour and 18-hour methods) that they more than compensated for the shortcomings in the assay design. In contrast, the frogs used by Laboratory 1241 were much less homogeneous in their innate susceptibility to digitalis as indicated by the composite slope values of 4.5 and 6.4.

Support for the hypothesis that the frogs themselves were responsible for the difference lies in the indication that Laboratory 1241 achieved perfect randomization in distributing the frogs into the various subgroups so that the flat slopes were in no way attributable to a heterogeneity of this sort. The data of these two collaborators not only illustrate the profound influence of a favorable slope on the precision of the assay, but they also demonstrate that the slope may vary greatly according to the assay technique and from one laboratory to another using the same technique. The advantage, however, of the 18-hour method over the 1-hour method in the hands of both of these collaborators is manifest in spite of the differences in slope each obtained.

To determine the general applicability of a standard curve for digitalis assays such as that provided in the British Pharmacopœia (1932) and suggested recently (15), the data in Table VI, cols. 5, 6 and 9–15, inclusive, have been compiled. The composite slope values obtained in the Second Comparison, in which only the 18-hour method was used, are included for comparative purposes.

The original data on which the values of cols. 9, 10 and 11 are based were examined by the χ_b^2 test for agreement between assays within the same laboratory for each Comparison as well as between Comparisons within the same laboratory. The data reveal highly satisfactory agreement of the slopes from one assay to another within a given Comparison. In only one case is there significant heterogeneity (Laboratory 1229, Second Comparison, where the χ_b^2 value is 26.9 with $n = 11$ and $P = 0.01$). Thus within the time interval required for completing the assays of a single Comparison (two to four weeks) the slope appears to be stable and the composite values listed in cols. 5, 6, 9, 10 and 11 for each laboratory may be looked upon as being "characteristic" (20) of the responses of frogs to digitalis by the method indicated under the conditions prevailing in the laboratory when the work was done.

Column 12 lists the composite 18-hour slope values for each laboratory submitting data in more than one of the first three Comparisons. In col. 13 are χ_b^2 values showing how well the two or three values agree among themselves. In 6 of the 10 cases in which a "between-Comparison" test for agreement of slope can be applied, the two (or three) values differ more than would be expected as a result of normal sampling variation. Although these data show that the slope of the dosage-effect relationship may vary within the confines of any single laboratory from time to time, they are wholly inadequate to tell whether the varia-

tion is seasonal in character. Finally, it should be reiterated that the assays were designed to reveal relative potency so that the information furnished regarding slope variations is not only limited but tends to underestimate their extent. With experiments designed to give better estimates of slope (*i. e.*, two doses, widely spaced so that positive effects of about 10 and 90 per cent, respectively, would be obtained), the true character and magnitude of the variation might be revealed.

The composite slope for all the 18-hour assays of the first three Comparisons is 9.18. However, the values going to make up this composite are highly heterogeneous (the chi-square value for the agreement of the 10 composites in col. 12 is 76.8) and hence no combined value may be regarded as representing adequately the slope observed in all the laboratories. This conclusion must be considered in the light of the fact that these data were obtained under conditions rigidly prescribed and controlled to a greater extent than is customary in ordinary routine digitalis assays. Such conditions should result in a minimum variation in slope; hence, there can be no doubt that the slope of the 18-hour dosage-mortality relationship varies greatly from one laboratory to another.

For the present, therefore, it seems prudent to retain a design which permits a calculation of the slope for each assay in contrast to the practice of using only one dose at each level of each preparation and the standard and interpreting the results from a standard curve. The simplest design meeting this requirement is that used in the Fifth Comparison which called for two doses of both standard and unknown.

The observed heterogeneity in slope suggests that the possibility exists for some laboratories to improve notably the precision of their assays by seeking the basis for the high slopes characteristic of other laboratories. The potential increase in efficiency whereby equal or greater precision could be attained by means of fewer frogs would result in considerable economy of animals and time. The data of Table VII make it appear that for the 18-hour assays, at least, there may be some correlation between the slope of the dosage-effect curve and the source of the frogs.

Columns 14 and 15 list for each laboratory the ratios between the observed 18-hour and 1-hour slope values for the First and Third Comparisons. As indicated by the average values for the two series, the ratios are generally higher in the First Comparison. While the ratio exceeds unity in every case, the advantage of the 18-hour method is clearly not always significant. It would therefore be difficult to choose between the two methods solely on the basis of the slopes of the characteristic dosage-effect relationships. There are, however, collateral considerations which support the tentative choice made between the two methods on the basis of the results of the first three Comparisons. However, later developments (21, 22) made a final choice unnecessary inasmuch as the frog was supplanted by the cat as the test object for the assay of digitalis.

TABLE VIII.—COMPARISON OF THE EXPECTED POTENCY VALUES WITH THOSE OBSERVED IN THE 18-HR. METHOD

Comparison	Expected Potency[a]	Observed Potency[a]	Difference ± s. e.	Inert marc, %[b]	Basis of Expected Potency
First	140.0	139.2 ± 2.1	−0.8 ± 2.1	28.6	Known dilution
Fourth	9.93 ± 0.18	9.79 ± 0.19	−0.14 ± 0.26	13.4	Third Comparison data
Fifth	10.0 ± 0.19	9.71 ± 0.14	−0.29 ± 0.25	11.6	Fourth Comparison data

[a] Observed potency for First Comparison indicates strength of Sample 1 as a percentage of that of Sample 2; for Fourth and Fifth Comparisons, values are International Digitalis Units.
[b] Expressed as per cent of inert marc in adjusted powder.

THE RELIABILITY OF THE 18-HOUR METHOD

In three of the Comparisons discussed above the relationship between the standard digitalis and sample being assayed was known either exactly or within calculable limits. Thus Table VIII lists, with other pertinent data, the expected potency values and those observed with the 18-hour method in these Comparisons.

The expected potency values for the Fourth and Fifth Comparisons are based on the weighted averages of the respective preceding Comparison. Thus the expected values are known only within the limits of the error associated with the averages from which they are derived.

It will be noted that the agreement between observed and expected is remarkably good in each instance. In two of the three cases the difference (fourth column, Table

VIII) is much less than its standard error; in the third case, the difference exceeds the standard error only slightly. The fact that observed potency is slightly lower than that expected in each case is noteworthy. Compared with the desired value of exactly 10 International Digitalis Units per gram, the result of the Fifth Comparison is significantly low by conventional statistical standards but from a practical viewpoint the shortage is quite unimportant.

While the calculated standard errors of the 18-hour weighted averages appear almost incredibly low for data based on a pharmacological reaction, the correlations in Table VIII reveal they are quite valid. Further evidence on this point was adduced in the Fifth Comparison in which seven collaborators carried out three complete assays on a single day, thereby providing duplicate data on two of the four extracts being assayed. The duplicate data on each extract have been calculated as a single assay and the standard error of the result so obtained has been compared with the deviation from 100 per cent, the potency value to be expected in assaying any extract against itself. In 10 of the 24 results the deviation from 100 per cent exceeds the standard error but in only one is the deviation more than twice the error, a proportion quite within the limits of normal sampling.

CONCLUSIONS

1. Both the 1-hour and 18-hour methods of assay of digitalis on frogs, when carried out as directed for this study, are capable of yielding potency estimates that agree with known potency relationships within the experimental error calculated from the data (First and Third Comparisons). In the three instances where the observed potency can be compared with the known potency, the weighted average 18-hour value differs by 1 to 3 per cent from the true value (First, Fourth and Fifth Comparisons).

2. The maceration procedure for obtaining the activity of digitalis powder in a form suitable for assay yields very uniform results (Second and Fifth Comparisons); a hot extraction procedure using 95 per cent alcohol as the solvent appears less satisfactory from the limited trial it was afforded (Second Comparison).

3. The slope of the dosage-effect relationship for digitalis in frogs by both the 1-hour and 18-hour methods is fairly stable within any one laboratory for limited periods of time (First and Third Comparisons). Although in some laboratories the slope by the 1-hour method is as high as that by the 18-hour method, most collaborators find a significantly higher slope by the latter procedure. The data indicate that the 18-hour slope varies significantly from time to time in some laboratories and highly significantly from one laboratory to another (all Comparisons). While no single slope value can be assigned as characteristic of either method, slopes of the order of 6 and 10 probits per unit increase in log-dose are most frequently observed in the 1-hour and 18-hour data, respectively. Thus assays on equal numbers of frogs generally yield a more precise result (lower calculated standard error) by the 18-hour method.

4. Considerable variation is noted in the dosage of digitalis used by different collaborators to produce comparable levels of effect; the difference is as great as 100 per cent in the 1-hour method and 50 per cent in the 18-hour method (First and Third Comparisons).

5. A two-dose procedure for the 18-hour method has been developed which gives an accuracy of ±20 per cent (Fifth Comparison).

REFERENCES

(1) Miller, L. C., THIS JOURNAL, 29 (1940), 339.

(2) Stenographic Transcript of U. S. P. Conference on Digitalis Assay held August 23, 1939, at Atlanta, Ga.; transcript available on loan from the U. S. Dept. of Agriculture Library, Washington, D. C. (Call No. 396 Am 35).

(3) Letters 1 and 2 on the U. S. P. (1939–1941) Digitalis Assay Study. (These letters and those mentioned in succeeding references were issued by the General Chairman of the U. S. P. Revision Committee to the collaborators during the course of the study.)

(4) Letter 6.

(5) Letter 5.

(6) Miller, L. C., Bliss, C. I., and Braun, H. A., THIS JOURNAL, 28 (1939), 644.

(7) Letter 1.

(8) Letter 3.

(9) Bliss, C. I., *Quart. J. Pharm. Pharmacol.*, 11 (1938), 192.

(10) Snedecor, G. W. "Statistical Methods," ed. 3, Collegiate Press, Inc., Ames, Ia.

(11) Fisher, R. A., "Statistical Methods for Research Workers," ed. 7, Oliver and Boyd, London, 1938.

(12) Letter 10.

(13) Chapman, C. W., THIS JOURNAL, 29 (1940), 337.

(14) Letter 18.

(15) Allmark, M. G., and Morrell, C. A., THIS JOURNAL, 30 (1941), 1.

(16) Letter 19.

(17) Letter 15.

(18) Letter 20.

(19) Miller, L. C., and Braun, H. A., *J. Pharmacol.*, 69 (1940), 295.

(20) Trevan, J. W., *Proc. Roy. Soc.* (London), B, 101 (1927), 483.
(21) Minutes of the U. S. P. Conference on Digitalis Assay held September 6, 1941, in New York, N. Y. A copy is available from the General Chairman of the U. S. P. Revision Committee, Philadelphia, Pa.
(22) Bliss, C. I., THIS JOURNAL, 33 (1944), 225.

Laxative Action in Mice of Tinnevelly and Alexandria Senna, and of Several Botanically Related Plants*

By I. W. Grote and Maribelle Woods

Senna consists of the dried leaflets of *Cassia acutifolia Delile*, known in commerce as Alexandria senna, sometimes called Egyptian senna; and of *Cassia angustifolia Vahl*, commercially known as Indian or Tinnevelly senna (1). In materia medica, senna is placed in the anthraquinone-containing group of vegetable purgatives which act on the large intestine, "and is one of the most efficacious drugs of this class" (2).

Alexandria senna is collected mostly from wild plants growing in the Nile Valley from Assouan to Kordofan. Usually it is shipped in loose bales or bags containing many broken leaves. For domestic sale in small packages, some whole leaves are separated, but the commercial grades consist largely of mixed or broken leaf, half leaf, fourth leaf, and siftings.

The greater amount of the commercial senna supply consists of Tinnevelly senna collected from *Cassia angustifolia* plants in the Tinnevelly section of southern India, grown originally from seed obtained from wild growth in Arabia. The cultivation, collection and grading of Tinnevelly senna is much more carefully done than with Alexandria senna. Most of the leaflets are received in this country in unbroken form, having been shipped in hard-pressed bales of approximately four hundred pounds each. These are graded in the country of origin as No. 1, No. 2 and No. 3. "The grading is done," according to a letter to the authors from S. B. Penick & Co., importers, "entirely by physical characteristics and by the size of the leaf. Color is not usually considered. For instance, even though a No. 1 is faded in color it would still be classified No. 1 because of its size. The actual size of the leaf included in each grade is variable and dependent somewhat on the crop conditions from year to year. The grading is not done by measurement but by sight."

In general, all three grades of Tinnevelly senna are cleaner and less likely to include brown leaves, stems and pods than is the case with Alexandria senna, although both drugs are remarkably clean and free from adulterants.

Notwithstanding the better and more uniform appearance of Tinnevelly senna, Alexandria senna traditionally is more highly regarded and usually brings a higher price than Tinnevelly. Both contain anthraquinone derivatives, and the statement is variously made that Alexandria senna contains more of these derivatives than does the Tinnevelly. According to Maurin, quoted by Trease (3), Alexandria senna contains 1.55 per cent of anthraquinone derivatives while Tinnevelly contains 1.35 per cent, the chief derivatives being aloe-emodin and rhein. Both derivatives exist in the free state and as glycosides and were isolated by Tutin in 1913 (4).

We were unable to find a comparison of the relative laxative action of Alexandria and Tinnevelly senna. Therefore, we conducted such an investigation, using the mice assay method of Geiger (5) with modifications by Hazleton and Fortunato (6) and with a few modifications by our own staff.

EXPERIMENTAL

The mice were kept in colony cages in a room in which the temperature and ventilation were controlled, and fed a constant diet of specially prepared mouse pellets. No essential difference was found between the laxative dose required for adult male and female mice, but the sexes were kept separated to avoid possible variations due to pregnancy complications. Mostly male mice were used in this work. The mice were weighed previous to dosage and were then placed in individual wire cages raised above an absorbent paper so protected by wire mesh as to prevent destruction by the animal. The animals used were mature mice weighing between 18 and 28 Gm. At the end of a thirty to

* Received Jan. 1, 1944 from the Research Laboratory of The Chattanooga Medicine Co., Chattanooga, Tenn.

forty-five minute observation period, mice showing no signs of soft stools or diarrhea characterized by fecal stains appearing through the underside of the paper were employed for the tests. The animals were not used more than once a week.

The senna test samples were prepared by grinding the leaflets to completely pass an 80-mesh sieve and were made into suspensions with boiling distilled water. One-half cubic centimeter doses of such suspensions were given the mice by stomach tube and the mice were then placed in individual wire cages elevated over paper as before and left overnight. Fecal stains on the paper from the animals showing laxative action were easily distinguished from urine stains by reading the stains on the underside of the paper. At least ten mice were treated with each of three dilutions of a Tinnevelly senna used as a standard, and usually twenty-five on each of three dilutions of the "test" senna on the same day. The dilutions were then adjusted on successive days so that preferably approximately 75 per cent, 50 per cent and 35 per cent positive laxative results were obtained, respectively on each of the three dilutions of the reference standard senna and the "test" material. The results were then interpreted according to standard procedure. The dosage required to produce laxation in 50 per cent of the animals is called the M. La. D_{50} At least a total of fifty animals was used on each of the three experimental doses for the "test" sennas. A 0.5-cc. dose of distilled water, under identical conditions, was found to be without effect.

In order to have a reference standard for comparison of laxative action, several pounds of exceptionally bright green Grade 1 Tinnevelly senna were ground to pass an 80-mesh sieve and packed carefully into small tightly sealed bottles. This reference standard has been used throughout our work for the past year, and has proved remarkably uniform in producing a laxative effect. A dose of 6.3 mg. produced a laxative effect in 54 per cent of 980 mice, while 8.3 mg. produced such an effect in 72 per cent of 570 animals, and a dose of 5.0 mg. produced a laxative effect in 39 per cent of 683 animals. Dosages of plus or minus 20 per cent above or below those necessary to produce a M. La. D_{50} effect are readily detected. The test therefore seems sufficiently sensitive and reproducible to be of a quantitative bioassay nature. The Tinnevelly senna used as a standard when bioassayed in another laboratory by this same method gave practically identical results. Mice seem to be slightly more sensitive to the action of senna during weather in which the barometer is rapidly changing, but no seasonal variation has been found and the animals treated with the reference standard senna during the tests herein reported check closely the results obtained during the entire one-year period.

Representative samples of Alexandria and Tinnevelly sennas were obtained from several sources and ground to pass an 80-mesh sieve, and were administered under conditions identical with those of the reference standard senna. All samples examined met the U. S. P. specifications for senna. The results of the animal tests are given in Table I.

Samples larger and smaller than the doses shown in Table I were also given in many cases, with results agreeing with those obtained from the more significant medial doses. To compute the M. La. D_{50}, the logarithms of the dose in milligrams were plotted against the percentage of response to laxative action and the M. La. D_{50} obtained by interpolation. Two thousand two hundred doses of the reference standard Tinnevelly senna demonstrated by statistical interpolation that a dosage of 5.9 mg. was required to produce a laxative effect in 50 per cent of the mice, and this dosage result has been called 100 per cent activity. Three thousand two

TABLE I.—LAXATION IN MICE PRODUCED BY DIFFERENT GRADES OF TINNEVELLY AND ALEXANDRIA SENNA

Test Material	Dose, 12.5 Mg.[a]			Dose, 8.3 Mg.[a]			Dose, 6.3 Mg.[a]			Dose, 5.0 Mg.[a]				
	Number of Mice	Positive Response	Positive Percentage	Number of Mice	Positive Response	Positive Percentage	Number of Mice	Positive Response	Positive Percentage	Number of Mice	Positive Response	Positive Percentage	Calculated Mg. Dose, M. La. D_{50}	Percentage of Ref. Standard Senna
Reference Standard Select No. 1 Tinnevelly	...	..	..	570	411	72	980	525	54	683	266	39	5.9	100
No. 1 Tinnevelly	50	45	90	75	27	36	75	22	29	50	12	24	8.8	67
No. 2 Tinnevelly	...	..	..	50	31	62	50	21	42	50	15	30	6.9	86
No. 3 Tinnevelly	50	45	90	125	79	63	125	70	56	75	25	33	6.1	97
5-Yr.-Old Tinnevelly No. 2 Leaves	...	..	..	50	38	76	50	30	60	50	17	34	5.8	102
5-Yr.-Old Tinnevelly No. 3 Broken Leaves	...	..	..	50	40	80	50	27	54	50	18	36	5.9	100
5-Yr.-Old Tinnevelly Nos. 1 and 2 Powdered Leaves	...	..	..	50	35	70	50	32	64	50	14	28	5.9	100
Whole Leaf Alexandria	130	85	65	130	40	31	130	13	10	...	...	..	10.4	58
Half Leaf Alexandria	50	33	66	50	21	42	50	11	22	...	...	..	9.4	63
Fourth Leaf Alexandria	...	..	..	75	57	76	75	51	68	150	65	43	5.3	111
Fine Siftings Alexandria	20	15	75	120	76	63	100	56	56	100	48	48	5.2	113
Mixed Leaf Alexandria	90	74	82	175	101	58	145	71	49	75	16	21	6.5	91
Siftings Alexandria	90	71	79	100	51	51	90	31	34	...	...	..	8.1	73

[a] Dose was suspended in 0.5 cc. distilled water.

hundred additional doses were given from the twelve samples of senna listed in Table I. The results may be summarized as follows:

An unselected No. 1 Tinnevelly senna was 67 per cent as active as the reference standard; a No. 2 Tinnevelly senna was 86 per cent as active; a No. 3 was 97 per cent as active. A mixed No. 1 and No. 2 Tinnevelly senna which had been powdered more than five years ago was 100 per cent as active as the reference standard. Another No. 2, over five years old, was 102 per cent; and a No. 3 over five years old, was 100 per cent of the standard. With the Alexandria senna samples, one of whole leaf was 58 per cent of the reference standard; a half-leaf grade was 63 per cent of the standard; a fourth-leaf grade was 111 per cent; a mixed leaf was 91 per cent, while two separate samples of siftings showed 113 per cent and 73 per cent of the reference standard strength.

If the six samples of each of the two species are added, a dose of 6.6 mg. of Tinnevelly and 7.4 mg. of Alexandria would be required to produce the same laxative action as that obtained from 5.9 mg. of the reference standard Tinnevelly.

COMPARATIVE LAXATIVE ACTIVITY OF SEVERAL SENNA-RELATED PLANTS

War shipping difficulties, threatening to decrease importations of Tinnevelly and Alexandria senna, suggested that our investigation be extended to include several of the domestic, botanically related Cassia plants. The genus *Cassia* is represented by at least five species which may be found growing wild in Tennessee; namely, *Cassia marilandica L.* (Maryland senna), *Cassia medsgeri Shafer* (wild senna), *Cassia chamaecrista L.* (partridge pea), *Cassia nictitans L.* (wild sensitive plant) and *Cassia tora L.* (low senna or coffee weed). Because of their immediate taxonomic relationships, leaves of *Cercis canadensis L.* (red bud) and of *Gleditsia triacanthos L.* (honey locust) were also included.

Except that *Cassia marilandica* is said by some authorities to have laxative properties, the scientific literature has but scant references to these wild "sennas" beyond describing their botanical characteristics and occasionally speculating as to possible laxative effects of some of them. Slama (7) made an excellent morphological study in 1934 of the first four of the Cassias just mentioned. *Cassia marilandica* was listed under this name as a drug in the United States Pharmacopœia from the first edition in 1820 through the 1873 edition, although it was listed separately from the official sennas and it was not indicated to be used as a source thereof or as of equal value. Its therapeutic action was understood to be "similar to the Alexandrian senna, but less active" (8), and scattered references can be found to its supposedly "cathartic acid" content. The dose, according to U. S. P. II, was one ounce of the dried leaf in infusion while the U. S. P. II dose of the official senna of 1830 *Cassia acutifolia* was one scruple to one dram, or not more than 10 per cent as much as was required of *C. marilandica*. It is doubtful if Maryland senna was ever much used, since Oldberg and Wall (9) in a contemporary commentary on the sixth revision of the Pharmacopœia stated: "We have vainly endeavored to obtain some of it on the market, which proves it is not now used, if it ever was."

EXPERIMENTAL

Sufficient material for the proposed laboratory study of *Cassia marilandica* was not obtainable from locally accessible wild growths, and large wild stands of it could not immediately be located elsewhere. An experimental crop of *C. marilandica*, sufficient for the purpose, was produced from rootstocks obtained from the Wayside Gardens of Mentor, Ohio, and divided to propagate approximately five hundred plants. These were set out in Chattanooga, Tennessee, in March, 1942. This material was thoroughly authenticated as *Cassia marilandica L.* by several well-known pharmacognosists. Within the months of July and August, 44 pounds of dried leaves were harvested from 458 plants.

A considerable supply of *Cassia tora*, *Cassia medsgeri*, *Cassia chamaecrista* and *Cassia nicititians* was obtained from wild stands near Chattanooga, and leaves from *Cercis canadensis* and *Gleditsia triacanthos* were similarly obtained and prepared by being allowed to dry spontaneously, ground and passed through an 80-mesh sieve. Table II shows the results of the administration of these seven plant leaves to mice as described in our comparative study of the laxative action of Tinnevelly and Alexandria senna.

RESULTS AND DISCUSSION

The five "wild sennas" and their two relatives were found to be inert in dosages up to ten times that of the reference standard senna which had been prepared from *Cassia angustifolia*, only a single positive result being obtained with one animal out of the hundreds employed in the study. A dosage greater than 50 mg. per animal could not be easily administered to mice by stomach tube. In the case of the *Cassia marilandica*, which traditionally was supposed to possess laxative property, a 50–50 admixture with reference standard *Cassia angustifolia* (Tinnevelly senna) was administered with less laxative effect than that obtained from the *C. angustifolia* content alone. Possible diverse effects of climate, age of leaf when harvested, and of soil characteristics on the laxative actions of the "wild sennas" were eliminated by the fact that *Cassia angustifolia* grown from seed planted in the same local area, cultivated, harvested and dried under similar conditions, possessed a laxative activity substantially equal to some of the imported official Tinnevelly senna.

Since "mice are not men," and in view of

the negative results obtained from administration of *Cassia marilandica* to the animals, two male human subjects in apparently normal health volunteered to take test doses of this one wild senna previously reported active (8). Dosages of 0.5 Gm. of *Cassia angustifolia* (Tinnevelly senna) had a marked laxative action on these two subjects, and dosages of 2.0 Gm. had a violent purgative action. Dosages ranging up to 5.0 Gm. of *Cassia marilandica* (Maryland senna) were without effect.

As a possible chemical verification, the well-known Borntrager reaction, as well as several recent modifications of it, were also carried out on the series of Cassia under investigation. This reaction consists of extracting an acid infusion of senna with ether or other solvent such as benzine, and then adding an alkali to the ether extract. Both of the official U. S. P. sennas tested give a distinct red color, but all the seven inert species tested yielded only varying shades of yellow without a tinge of red.

TABLE II.—EFFECTS OF THE ADMINISTRATION OF CERTAIN SENNA-RELATED PLANT LEAVES TO MICE

Test Material	Mg./Dose	Number of Mice	Number Showing Laxative Action	Percentage Positive	Approx. Percentage of Ref. Standard Activity
Distilled water	0	50	0	0	0
Reference Standard Leaflets of *Cassia angustifolia* (Tinnevelly senna)	8.3	250	171	68	100
	6.3	430	223	52	
	5.0	350	137	39	
	4.2	200	47	24	
C. marilandica	50.0	50	0	0	Below 10%
	25.0	40	1	2.5	
	12.5	40	0	0	
	8.3	10	0	0	
	6.3	10	0	0	
	5.0	10	0	0	
C. marilandica plus *C. angustifolia* Reference Standard 50–50 mixture	25.0	50	39	78	80–90 based on *C. angustifolia* alone
	12.5	50	21	42	
	8.3	50	7	14	
C. tora	50.0	50	0	0	Below 10%
	25.0	30	0	0	
	12.5	10	0	0	
C. chamaecrista	50.0	50	0	0	Below 10%
	25.0	20	0	0	
C. nictitans	50.0	50	0	0	Below 10%
	25.0	30	0	0	
C. medsgeri	50.0	50	0	0	Below 10%
	25.0	20	0	0	
	12.5	20	0	0	
	8.3	20	0	0	
	6.3	20	0	0	
Cercis canadensis	50.0	50	0	0	Below 10%
	25.0	20	0	0	
	12.5	20	0	0	
	8.3	20	0	0	
Gleditsia triacanthos	50.0	30	0	0	Below 10%

SUMMARY AND CONCLUSIONS

1. From experimental administration of the leaflets of *Cassia acutifolia Delile* (Alexandria senna) and *Cassia angustifolia Vahl* (Tinnevelly senna) to mice, the laxative actions of the two kinds of senna do not appear to differ widely.

2. Variations between different test samples within each kind of senna were as great as between representative samples of each of the two kinds of senna.

3. Samples of *Cassia angustifolia Vahl* (Tinnevelly senna) from commercial stocks which had been stored in warehouses five years or possibly longer showed no appreciable loss of activity.

4. Five native "wild sennas" and two related plants in ten times the dosage required of the official sennas were without demonstrable laxative activity when administered to mice.

5. One "wild senna" (*Cassia marilandica*) was found to be inert in two human subjects when administered in dosages up to 5 Gm. while a dose of 0.5 Gm. Tinnevelly senna caused consistently a laxative action in the same subjects.

REFERENCES

(1) "United States Pharmacopœia XII," Mack Printing Company, Easton, Pa., 1942, p. 402.
(2) "Useful Drugs," ed. 11, American Medical Association, Chicago, Ill., 1938, p. 182.
(3) Trease, George Edward, "A Textbook of Pharmacognosy," ed. 3, 1939, p. 498.
(4) Trease, George Edward, *Ibid.*, p. 498.
(5) Geiger, E., THIS JOURNAL, 29 (1940), 148.
(6) Hazleton, L. W., and Fortunato, F., *Ibid.*, 31 (1942), 60.
(7) Slama, Frank J., *Ibid.*, 23 (1934), 550.
(8) "United States Pharmacopœia II"," 1830, p. 26.
(9) Oldberg, Oscar, and Wall, Otto A., "A Companion to the United States Pharmacopœia, 1884, p. 916.

A Phytochemical Investigation of *Hermidium Alipes*[*,†]

By Donald W. Buelow[‡] and Ole Gisvold[§]

Hermidium alipes (S. Watson) is a North American plant of the Nyctaginaceae or Four O'Clock family and is found on the foothills of Utah, Nevada and California (1). It is a perennial glabrous herb, five to twelve inches high, with entire, opposite, short-petioled leaves. The flowers occur in head-like clusters on the ends of terminal or axillary peduncles. The clusters are six to eight flowered, each flower subtended by a large ovate leathery bract, and the short pedicels united to the midveins of the bract. The most common member of the family is *Mirabilis jalapa* which is widely cultivated as a flowering plant.

Hermidium alipes is worthy of a phytochemical investigation because a fluidextract of the plant when injected into the blood stream of experimental laboratory animals produces a marked rise in blood pressure. The plant, therefore, must contain one or more blood pressor substances. The rises produced are similar to those of epinephrine; however, the duration of action is longer than that of epinephrine.

A phytochemical and pharmacological study of *Hermidium alipes* was previously reported (2) in which all attempts to isolate the active principle had failed. A careful study of the techniques employed in this report led to the conclusion that the problem was worthy of reinvestigation. This conclusion was well founded by the isolation of 3,4-dihydroxyphenylethylamine in the free state.

Figure 1 shows the characteristic response obtained by the intravenous injection of a fluidextract of *Hermidium alipes* in the femoral vein of a rabbit. Figure 2 shows the characteristic response obtained from crystalline material (subsequently identified as 3,4-dihydroxyphenylethylamine) obtained from *Hermidium alipes*. Figures 3 and 4 are a quantitative comparison (3) of 3,4-dihydroxyphenylethylamine to epinephrine and indicate that the former has about one-fortieth the activity of the latter. Figure 5 is a similar comparison in which the drum holding the tracing has merely been reduced in speed to show more closely the quantitative and qualitative relationships.

The plant material for this study consisted of roots collected near Reno, Nev., by the Division of Plant Exploration and Introduction, Bureau of Plant Industry, United States Department of Agriculture.

EXPERIMENTAL

The plant material used was part of that which had been used in the original investigation of this plant. The dried roots had been ground to a No. 60 powder and had been stored in tightly closed tin containers.

Preliminary Experimental.—The solubility of the active principle was determined by the preparation of various extracts of the plant. Each of these extracts was tested for its pressor effect by noting the rise in blood pressure obtained by injection into the femoral vein of a rabbit. One hundred grams of the plant material was successively extracted in a Soxhlet apparatus with various solvents. Petroleum ether, diethyl ether, chloroform, ethyl acetate, and 95% ethanol extracts were made. The solvents were removed under reduced pressure and the residues digested with 70% alcohol. When these filtered solutions were injected intravenously into a rabbit, only the original 95% ethanol extract exhibited activity.

The marc which remained after extraction with the above solvents was moistened with 95% ethanol containing 0.5% hydrochloric acid. It was allowed to macerate for twenty-four hours and was then

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percolated with the above solvent. This acid-alcohol extract, when neutralized at the time of injection, was also active.

Exposure of the acid-alcohol and 95% ethanol extracts to the air and heat resulted in the loss of much of the activity. Removal of the solvents from these extracts yielded residues which were soluble in water. These aqueous solutions were active when tested.

Fifty grams of the drug was heated with 200 cc. of water on the steam bath for thirty minutes. The aqueous solution when filtered from the marc had marked activity indicating that the active principle could be extracted from the drug with water. The marc was also boiled with a 0.2% acetic acid solution. This acid solution, when neutralized with sodium bicarbonate prior to injection, was found to be very active.

In an effort to find some solvent other than alcohol or an acid-alcohol mixture in which the active constituent might be soluble, a portion of an alcohol extract was concentrated to a syrup under vacuum, in a carbon dioxide atmosphere. This syrup was boiled with dioxane, benzene and amyl alcohol. The solvents were removed from the solutions under reduced pressure and the residues dissolved in water. These aqueous solutions were inactive when tested, indicating that the active principle was not soluble in the above solvents.

One hundred grams of the drug was macerated for forty minutes with 100 cc. of ammonium hydroxide. It was quickly dried in a current of warm air, packed in a Soxhlet and successively extracted in a Soxhlet apparatus with diethyl ether, chloroform and 95% ethanol. These extracts were inactive when tested.

Maceration and extraction of a portion of the plant material with Prollius' fluid gave a negative test for alkaloids when tested with the usual alkaloidal reagents.

The active 95% ethanol extract was very sensitive to sodium bicarbonate, sodium carbonate, ammonium hydroxide and sodium hydroxide test solutions which caused the formation of a black precipitate. Neutralization of the alkali failed to dissolve the precipitate or restore the activity. Ferric chloride test solution always produced an emerald-green color when added to an active extract. The use of strong alkali was avoided in subsequent work so that the activity of the extracts would not be destroyed. When an aqueous extract was exposed to the air for several days it also darkened and a black precipitate was formed. It was apparent that this darkening was due to oxidation.

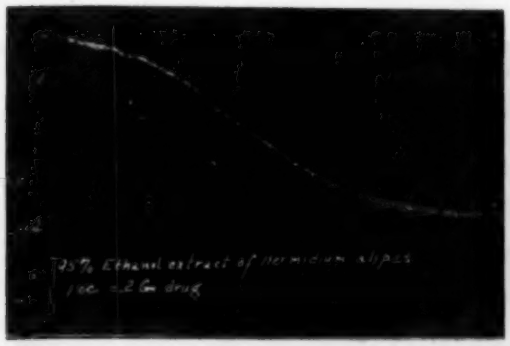

Fig. 1. Pressor response obtained from a 95% ethanol extract of *Hermidium alipes* by intravenous injection into a rabbit.

Fig. 2. Pressor response from 4 mg. of the crystals of *Hermidium*.

Fig. 3. Pressor response obtained by injection of 0.16 cc. of epinephrine hydrochloride into a dog.

The acid-alcohol extracts obtained after continuous extraction of the drug with 95% ethanol were light red in color and were considered to be relatively free of extraneous matter, such as sugars, proteins, inorganic salts, tannins, and the like, which would otherwise be present with the active constituent. Their absence should facilitate the isolation of the active pressor principle from a concentrated acid alcoholic extract even though considerable amount of the active principle had been removed in the 95% alcohol extraction.

tate was formed and centrifuged from the supernatant liquid.

The precipitate was collected on a suction filter and washed free of lead acetate with methanol. The lead precipitate was suspended in methanol and acidulated with concentrated hydrochloric acid. The dark red supernatant liquid was freed from the lead chloride precipitate and concentrated under vacuum to a thick syrup. This syrup was mixed with a small amount of 95% ethanol and placed in the refrigerator. After several hours it contained a

Fig. 4. Pressor response from 0.64 cc. of 3,4-dihydroxyphenylethylamine hydrochloride solution (1:1000) equivalent to 0.16 cc. of epinephrine hydrochloride solution (1:10,000).

ACIDULATED ALCOHOL EXTRACTION

Twenty-four hundred grams of finely powdered drug, which had previously been extracted with petroleum ether to remove the fat, was continuously extracted in a large metal percolator with 95% ethanol. The drug was then dried and extracted in the usual manner after a twenty-four hour maceration period with 95% ethanol containing 0.5% hydrochloric acid. The light red percolate was concentrated under reduced pressure in the presence of carbon dioxide and each liter of percolate concentrated to a volume of 250 cc. The concentrated solutions were placed in beakers exposed to the air and upon spontaneous evaporation deposited crystalline material. The crystals were removed by filtration and washed free of red coloring matter on the filter with 95% ethanol. A tan crystalline material (0.5 Gm.) resulted which contained inorganic matter. Upon recrystallization from hot 95% ethanol, most of the inorganics were removed and tan, platelike crystals resulted. The crystals produced an active pressor response when tested. (See Fig. 2.)

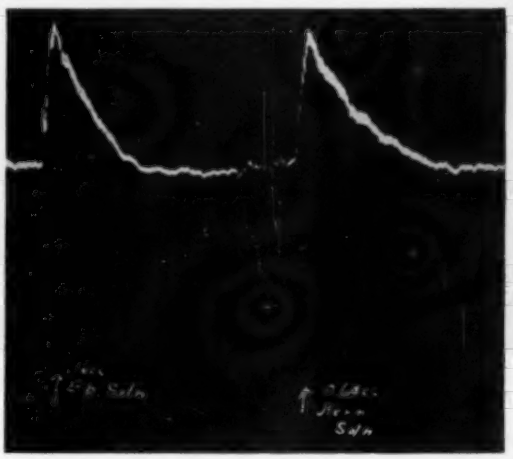

Fig. 5. Equivalent pressor responses from the solutions.

ATTEMPTED ISOLATION OF PRESSOR PRINCIPLE BY THE MODIFIED LEAD METHOD

A liter of the acidified-ethanol percolate obtained above was concentrated under reduced pressure to a thick syrup and poured into 200 cc. of distilled water. The solution was filtered, the dark red filtrate concentrated to a syrup under vacuum and dissolved in 125 cc. of methanol. The methanol solution was treated with a 20% solution of lead acetate in methanol. A tan, finely divided precipi-

solid mass of rosette-like crystals. This syrupy mass slowly darkened upon exposure to the air. Since the dark red color was intensified it was believed due to oxidation. This material was active when tested but the crystals could not be separated from the syrup.

LARGE-SCALE EXTRACTION

Preliminary experimental work had shown that

the direct acid-ethanol extraction of the defatted plant material was the best method to obtain the pressor principle. This method was therefore used to prepare sufficient material for subsequent analytical work.

Twenty-one hundred grams of the ground plant material, previously extracted with petroleum ether, was placed in a large glass percolator and extracted after maceration for twenty-four hours with 95% ethanol containing 0.5% hydrochloric acid. The acidity of the total extractive was controlled by using 5900 cc. of acidified ethanol for the maceration and beginning percolation of the drug. The extraction was then continued with 95% ethanol until the percolate was nearly colorless and no longer gave a green color with ferric chloride test solution.

Isolation of Crystals.—The total extractive was combined and concentrated to a syrup under reduced pressure in the presence of carbon dioxide. The resulting 150 cc. of syrup was mixed with 50 cc. of absolute ethanol and placed in the refrigerator overnight. Glistening plates of crystals formed in the syrup and were removed by centrifuging after the addition of 400 cc. of cold 95% ethanol. The crystals were washed free of the red color on the suction filter with 95% ethanol and dried on the filter. Four and one half (4.50) grams of tan crystalline material resulted. The filtrate and alcohol washing were combined and again concentrated and treated as described above. This second crystallization yielded 6.7 Gm. of crystals. The crystals were combined and purified by repeated recrystallization from hot 95% ethanol. After five or six recrystallizations from this solvent, light tan platelets were obtained. The hot ethanol solutions in each case had to be quite concentrated. If they were too dilute, the material would not crystallize when the solution was cooled.

Physical Examination of Crystals.—The compound, purified by crystallization from 95% ethanol, was obtained in the form of needles and plates. It crystallized as plates when a hot concentrated ethanol solution was cooled rapidly; or as needles when a more dilute solution was cooled slowly and allowed to evaporate spontaneously. The material usually possessed a slight tan color. Upon ignition, it burned with a smoky flame without leaving a residue. After drying the crystals in a vacuum desiccator over sulfuric acid and heating them in an evacuated Abderhalden drying pistol charged with phosphoric anhydride for one and one-half hours at 100° C., they had lost no weight and melted constantly at 241° C. (uncorr.) with decomposition. The compound gradually began to darken just below 200° C., and was black when melted. An aqueous solution of the compound was optically inactive. It was soluble in water, methanol and hot 95% ethanol; and insoluble in ether, chloroform, petroleum benzin, benzene and toluene. It was soluble in dilute alkalies. A sodium hydroxide solution of the crystals immediately assumed a red color. It was soluble in cold concentrated sulfuric acid. An aqueous solution of the crystals gave the following color reactions: an emerald-green color was produced when a drop of ferric chloride test solution was added. When a dilute solution of sodium carbonate was added drop by drop and the mixture shaken, the solution gradually became dark green, gray-green, blue, then purple, violet, and finally a red color. When this alkaline solution was acidified with dilute hydrochloric acid added drop by drop, the color changes occurred in the reverse order. When 1 cc. of ammoniacal silver nitrate test solution was added to a very dilute solution of the crystals a black color was produced immediately and after a few minutes a black precipitate formed. A few milligrams of the crystals were dissolved in 2 cc. of water and a few drops of Millon's reagent added. A red color developed in a few seconds without the application of heat to the solution. The addition of a few drops of sodium carbonate test solution to an aqueous solution of the compound almost immediately produced a light red color. After a short time a black precipitate was formed. The addition of a few drops of a 5% solution of sodium bicarbonate to a dilute aqueous solution of the compound produced a black insoluble precipitate after a short time. Ammonia test solution gave the same results as those obtained with sodium carbonate. The addition of silver nitrate test solution to an aqueous solution of the crystals gave a white curdy precipitate that was insoluble in dilute nitric acid. Oxidation of the crystals by means of acid and potassium iodate gave a deep cherry-red colored solution which became yellow upon dilution. Analysis for elements (4), following fusion with sodium, indicated the presence of nitrogen and chlorine. Application of the Hinsburg test (5) to a portion of the crystals indicated the presence of a primary amine because the reaction product with benzenesulfonyl chloride was soluble in alkali.

It can be concluded from the preceding qualitative tests that the crystalline pressor principle is the hydrochloride of a primary amine that contains a catechol group. All attempts to prepare the free base in the crystalline form failed.

Derivatives.—Attempts to prepare a crystalline acetyl derivative failed, although much time was expended in these attempts.

Methylation of the crystals by means of diazomethane or by means of dimethyl sulfate and alkali, and modifications thereof, yielded only an oily product.

A crystalline benzoate was first prepared (7) by means of dry pyridine and benzoyl chloride at room temperature. The excess benzoyl chloride was destroyed by mixing the reaction mixture with 10% cold ammonium carbonate solution. The derivative was recrystallized twice from hot isopropyl alcohol; m. p. 140° C. The same derivative was prepared by the Schotten-Baumann method using sodium hydrosulfite to prevent oxidation. This procedure proved more effective than the former. The benzoate was soluble in methanol, ethanol, benzene, chloroform and ether; and insoluble in water, dilute sodium hydroxide and dilute hydrochloric acid. It failed to give a color reaction with ferric chloride test solution.

A crystalline picrate (6) was prepared as follows: A small amount of the crystals was dissolved in 2 cc. of distilled water to which was added dropwise a saturated aqueous solution of picric acid until no more yellow precipitate was obtained. The yellow precipitate was collected and the product, when crystallized from hot water, yielded fine yellow needles; m. p. 189° C.

A crystalline styphnate was prepared in essentially the same way as the picrate. Instead of obtaining a yellow precipitate, the styphnate separated very shortly in the form of orange needle-like crystals. Recrystallization from hot water gave red-orange needle-shaped crystals; m. p. 206° C.

Anal.—Calcd. for $C_8H_{11}O_2N \cdot HCl$: C, 50.60; H, 6.36; N, 7.34; Cl, 18.73. *Found*: (av.) C, 50.65; (av.) H, 6.40; (av.) N, 7.16; (av.) Cl, 18.77. *Calcd.* for tribenzoate, $C_{29}H_{23}O_5N$: C, 74.81; H, 4.98. *Found*: (av.) C, 74.84; (av.) H, 4.89.

The chlorine content was determined gravimetrically as follows: Weighed samples were dissolved in water acidulated with nitric acid and an excess of 5% silver nitrate solution added. The mixture was stirred and allowed to stand for five minutes. The precipitated silver chloride was col-

lected in a fritted glass crucible, washed free of acid and dried to constant weight in the oven at 80° C.

A minimal molecular weight calculated from the above analytical data for one chlorine and one nitrogen agrees quite closely with the formula given under Analysis, *i. e.*, $C_8H_{11}O_2N \cdot HCl$.

The above data agree with that reported in the literature (8) for 3,4-dihydroxyphenylethylamine.

ISOLATION OF THE STEROL FRACTION

A light brown semisolid fat with a characteristic aromatic odor was obtained from a petroleum ether extract of several kilograms of the ground plant. The fat was saponified by heating on the steam bath for six hours with an excess of potassium hydroxide in 70% ethanol. The alcohol was removed by heating on the steam bath. The residue was dissolved in water and extracted with ether in a continuous extraction apparatus. The ether solution was washed with water, dried and the ether removed. The residue was digested with hot methanol from which plate-like crystals separated upon cooling. Several recrystallizations from ethanol yielded colorless plate-like crystals that melted at 135.5–136° C. These crystals gave a positive Liebermann-Burchard color test characteristic of sterols. The specific rotation of the sterol in chloroform in a concentration of 2.21 Gm. per 100 cc. was $[\alpha]_D^{25} -19.45$.

The sterol fraction when acetylated with hot acetic anhydride in pyridine yielded a crystalline acetate that melted at 119–120° C.

ISOLATION OF SUCROSE

Spontaneous evaporation of the ethanol extract of the drug, that was obtained by continuous extraction, yielded large cubical crystals. These were washed with alcohol to remove the syrup that adhered to them. They were crystallized from hot methanol after decolorization with charcoal. Several crystallizations from methanol gave a pure product that melted at 175–176° C. A mixed melt with pure sucrose showed no depression. It failed to reduce Fehling's solution before hydrolysis, but gave a positive reduction test after hydrolysis. The specific rotation of this compound was $[\alpha]_D^{25} +65.7$.

SUMMARY

1. 3,4-Dihydroxyphenylethylamine has been isolated as the crystalline hydrochloride from *Hermidium alipes*. This compound accounts in part at least for the blood pressure activity of this plant. The following crystalline derivatives have been prepared: a picrate, a tribenzoate and a styphnate, the last named being a new derivative.

This compound has one-fortieth the pressor activity of epinephrine.

2. A sterol fraction, m. p. 135.5–136° C., $[\alpha]_D^{25} -19.45$, acetate 119–120° C., was isolated from the nonsaponifiable portion of the petroleum ether extract.

3. Sucrose was isolated in its crystalline form. It was identified by its melting point, mixed melt with pure sucrose and specific rotation.

REFERENCES

(1) Jepson, W. L., "A Manual of the Flowering Plants of California," Williams Printing Company, San Francisco, Calif., 1925, p. 335.

(2) Walden, C. H., and Jenkins, G. L., *Pharm. Arch.*, 13 (1942), 65.

(3) Bieter, R. N., and Troxil, E. B., Personal Communication (1943).

(4) Kamm, O., "Qualitative Organic Analysis," ed. 2, John Wiley and Sons, Inc., New York, 1932, p. 133.

(5) Shriner, R. L., and Fuson, R. C., "The Systematic Identification of Organic Compounds," John Wiley and Sons, New York, 1935, p. 25.

(6) *Ibid.*, p. 140.

(7) Kitasato, Z. and Robinson, R., *J. Chem. Soc.* (1932), 786.

(8) Mannich, C. and Jacobson, W., *Ber.*, 43 (1909), 189; Barger, G. and Ewins, A. J., *J. Chem. Soc.*, 97 (1910), 2253; Guggenheim, M., *Helv. Chim. Acta*, 88 (1913), 276; Schopf, C. and Bayerle, H., *Ann.*, 513 (1934), 190; Schmalfuss, H. and Heider, A., *Biochem. Z.*, 236 (1931), 226.

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

8-Mercapto quinoline
2-Amino-4-methyl pyrimidine, 250 Gm.
l-Desoxy ephedrine hydrochloride
Guvacine or guvacoline (1,2,5,6-tetrahydronicotinic acid and methyl ester), 25 Gm.
α-Methyl stilbene
l-((*N*-Methylol stearic acid amido)methyl) pyridinium chloride
l-*N*-Ethyl ephedrine hydrochloride
l-((*n*-Nonadecanoic acid amido)methyl) pyridinium chloride
Ormosine
Stachydrine
Thymine (2,4-dihydroxy-5-methyl pyrimidine)
Tetraiodo pyrrole (iodol), 5 Gm.
l-((Thio octadecanoxy)methyl) pyridinium chloride
l-((*p*-Toluene sulfamido)methyl) pyridinium chloride
l,l,l-Tribrom tertiary butyl alcohol (Brometone)
Thio alcohols, of 8–18 carbons, pri., sec., or tert
2-Thio uracil
8-Nitroso quinoline

A Phytochemical Study of *Eriodictyon Angustifolium*, Nuttley*,†

By Willard J. Hadley‡ and Ole. Gisvold

The plant material used in the phytochemical investigation of *Eriodictyon angustifolium* was obtained from the Indian Drug Plant Project in Reno, Nev., Division of Plant Industry of the U. S. Department of Agriculture.

Eriodictyon californicum has interested physicians, chemists and pharmacologists for a long time. Plants of the genus *Eriodictyon* have had a reputation among the Indians and early Spanish settlers of California as a sure cure for consumption. The Pacific Pharmacopœia claimed that *Eriodictyon* was a specific for colds, asthma and grippe and was highly valued as a blood purifier and even a cure for rheumatism.

Fluidextract of Eriodictyon, Aromatic Elixir of Eriodictyon and Aromatic Syrup of Eriodictyon were official in the National Formulary of 1888. The syrup and the elixir were intended as a vehicle for quinine and other bitter remedies. In this use it has the properties masking the bitter taste of quinine. Fluidextract of Eriodictyon was official in the U. S. P. XI. Aromatic Syrup of Eriodictyon was official in the National Formulary VI.

The resinous material present in *Eriodictyon* is claimed (1) to be a substance which has the property of masking taste of quinine, strychnine, etc. The resinous material forms loose combinations with quinine, strychnine, etc., which can be broken up by very dilute acid or alkali and would, therefore, be effective when taken internally.

Szent-Györgyi (2) observed an effect on capillary permeability which he called "vitamin P" activity. He thought that the "vitamin activity" in "citrin," a crude flavanone fraction from lemon peel, was due to the presence of an eriodictyol glycoside. Citrin has been shown to be a mixture of eriodictyol and hesperidin. Recently, Higby (3) concluded that "An accumulation of evidence indicates that hesperidin in soluble form can function as vitamin P."

Nine species of *Eriodictyon* in all have been identified, but only the *E. californicum* has heretofore been subjected to a chemical investigation. Power and Tutin (4) and Tutin and Clewer (5) isolated several phenolic compounds, some hydrocarbons and a volatile oil from *E. californicum*. Of these constituents, only the flavones eriodictyol and homoeriodictyol have interested later investigators (6, 7, 8).

The prime object of this investigation was to determine the nature of the phenolic constituents of *Eriodictyon angustifolium*.

EXPERIMENTAL

The plant material consisted of brown and greenish yellow leaves and stems; the latter were very hard and tough. It was covered quite completely with the glistening, varnish-like surface exudation which is characteristic of the *Eriodictyon* species. The plant had a very aromatic odor, moderately pungent and somewhat camphoraceous. The taste was bitter and somewhat sharp. The plant material was ground to a No. 20 powder which was used for the subsequent experimental work.

Drug Constants.—The vegetable drug "constants" of the Pharmacopœia (9) were determined and the average percentage values tabulated as follows:

Moisture	7.98
Total ash	7.89
Acid-insoluble ash	0.64
Benzin-soluble extractive	4.06
Ether-soluble extractive:	
Volatile	1.66
Nonvolatile	15.73
Ethyl acetate-soluble extractive	21.65
Alcohol-soluble extractive	28.37
Dil. alcohol-soluble extractive	40.40
Water-soluble extractive	48.93

Qualitative Analysis of the Ash.—Aluminum, calcium, magnesium, sodium, potassium cations and sulfate silicate, fluoride, nitrate, and acetate anions were found (10).

Alkaloids.—A Prollius' fluidextract failed to give a positive test with the usual alkaloidal reagents. These results were confirmed by the Stas-Otto method (11).

Glycosides.—A positive test for the presence of glycosides was obtained by the Stas-Otto method.

Successive Extractions.—Five hundred grams of the powdered drug was placed in a Soxhlet and extracted successively with petroleum ether, ether, chloroform, ethyl acetate and ethyl alcohol. The percentages of the dried extracts are tabulated as follows:

Petroleum ether	6.45
Ether	12.90
Chloroform	1.20
Ethyl acetate	8.46
Ethyl alcohol	4.56

* Received Sept. 2, 1944, from the School of Pharmacy, University of Minnesota, Minneapolis, Minn.

† An abstract of a thesis submitted to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

‡ Assistant Professor, College of Pharmacy, Howard University, Birmingham, Ala.

Direct Alcoholic Extraction of the Plant.—Seventeen thousand two hundred and nineteen (17,219) grams of the plant material yielded 26.16% of an alcoholic extract that resembled a heavy, greenish black paint containing considerable congealed and lumpy material. This extract was used for the subsequent chemical investigations.

Volatile Oil.—The extract was subjected to steam distillation and 6.6 cc. (0.036%) of a volatile oil was obtained which was very pale yellow and had a spicy odor that was characteristic of the drug. Because of the small amount of oil obtained, its chemical investigation was quite limited to the following values: $[\alpha]_D^{25}$ 0.138; specific gravity 1.4679 at 20° C.; acid value 5.13; and ester value 116.8. Qualitative tests indicated the absence of aldehydes, ketones and phenols. It did not absorb bromine, but gave a positive test for active hydrogen.

Isolation of Salicylic Acid.—The aqueous liquid recovered from the nonvolatile solids was extracted with ether. The ether in turn was fractionally extracted with 10% aqueous sodium carbonate which upon acidulation yielded a resinous material. This resinous material from the latter fractions was dissolved in ether and after spontaneous evaporation only a resinous mass was obtained from which minute crystals protruded above the surface of the solid material. Attempts to effect a separation of this crystalline material by mechanical methods or by fractional crystallization from various solvents failed. It was impregnated upon sand and subjected to vacuum sublimation and some pure crystalline material was obtained which melted at 156–157° C. It was insoluble in water, soluble in bicarbonate and gave a violet color with ferric chloride. It did not depress the melting point of a sample of pure salicylic acid.

Anal.—Calcd. for $C_6H_6O_3$: C, 60.87; H, 4.38. Found: (av.) C, 60.91; (av.) H, 4.50.

Isolation of 3-Hydroxy-orthotoluic Acid.—The resinous material obtained from the first fractions (see Isolation of Salicylic Acid) was dissolved in ether. Upon spontaneous evaporation of the solvent at least two types of crystals were obtained, which were mixed with the resinous phenolic material. By washing this material with warm diluted alcohol several grams of a yellow-brown amorphous residue was obtained. It was very sensitive to oxidation by air and ether containing peroxides. It was dissolved in peroxide-free ether and a small amount of a yellow crystalline powder was obtained upon spontaneous evaporation of the solvent. It melted at 217° C. and gave a black precipitate with ferric chloride. It was thought to be impure homoeriodictyol and the amount was too small to warrant further characterization.

Attempts to obtain the second crystalline material in a pure state by fractional crystallization from various solvents failed. It was then mixed with sand and subjected to vacuum sublimation. About 2.5 Gm. of a white crystalline material was obtained. It melted at 165° C. It was insoluble in water but soluble in 5% sodium bicarbonate. It gave a violet coloration with ferric chloride test solution. Acetylation with acetyl chloride in pyridine yielded an acetate, m. p. 129.

Anal.—Calcd. for $C_9H_8O_3$: C, 62.73; H, 5.26. Found: (av.) C, 61.89; (av.) H, 5.19.

Molecular weight, Rast.

Anal.—Calcd. for $C_9H_8O_3$: 152.06. Found: 152.74, 151.84.

Neutralization equivalent.

Anal.—Calcd. for $C_9H_8O_3$: 152.06. Found: 149.93, 148.95.

The above data agree with that reported in the literature for 3-hydroxy-orthotoluic acid.

Identification of l-rhamnose.—Treatment of a portion of a water-soluble nonvolatile extract in the usual manner for the preparation of osazones yielded a derivative which melted at 177° to 180° C. One cubic centimeter of this aqueous solution was boiled with 10 cc. of concentrated hydrochloric acid and 1 cc. of aniline acetate. A permanent yellow color was obtained which is characteristic of methyl pentoses. The melting point of the osazone of *l*-rhamnose melts at 182° C.

Isolation of Homoeriodictyol.—The phenolic content of the solid nonvolatile material that had been subjected to steam distillation had evidently been degraded by this drastic treatment and, therefore, was not used for further phenolic studies.

Two thousand grams of the ground plant was percolated to exhaustion with ether. The extract was concentrated to 2 liters of solution which was brownish green in color. The ether solution was then extracted with cold 5% ammonium carbonate. Acidulation yielded a tarry resinous matter from which attempts to obtain any crystalline material failed. The ether solution was then fractionally extracted with cold aqueous 5% sodium carbonate. The first fractions upon acidulation yielded a light yellow precipitate, whereas the latter were orange-red and contained no solid material.

The light yellow precipitate was dissolved in hot water and upon cooling, 57 Gm. of a yellowish finely crystalline material separated. Upon recrystallization from aqueous acetic acid, 19.5 Gm. of an almost colorless crystalline powder was obtained, m. p. 224° C. It was insoluble in 5% aqueous sodium bicarbonate, but soluble in 5% aqueous sodium carbonate. It gave a brownish red color with ferric chloride test solution. It was not very soluble in water.

Anal.—Calcd. for $C_{16}H_{14}O_6$: C, 63.03; H, 4.98. Found: (av.) C, 63.08; (av.) H, 4.93.

Calcd. for $C_{15}H_{11}O_5(OCH_3)$: 10.23% methoxyl. Found: (av.) 10.35.

The above data agree with that reported in the literature (12) for homoeriodictyol.

The fractions obtained with sodium carbonate that were most soluble gave indications of the presence of eriodictyol.

SUMMARY

Homoeriodictyol (4′,5,7-trihydroxy-3′-methoxyflavanone) has been isolated from the ether-soluble extract of *Eriodictyon angustifolium*. Qualitative tests indicated that eriodictyol was also present in the ether extract; however, attempts to isolate it failed.

A very small amount of a volatile oil, salicylic acid, 3-hydroxyorthotoluic acid and rhamnosazone were isolated from an alcoholic extract of the plant.

The plant constants of the Pharmacopœia were determined.

Successive extractions of the plant were made with the following solvents: petroleum ether, ether, chloroform, ethyl acetate and ethyl alcohol.

Alkaloids were shown to be absent.

Qualitative tests indicated the presence of glycosides.

A qualitative analysis of the inorganic

constituents of the ash showed the presence of aluminum, calcium, magnesium, sodium, potassium, sulfates, silicates, fluorides and nitrates.

REFERENCES

(1) Fantus, B., *et al.*, THIS JOURNAL, 22 (1933), 323.
(2) Szent-Györgyi, A., *Nature*, 138 (1936), 27, 798.
(3) Higby, R. H., THIS JOURNAL, 32 (1943), 74.
(4) Power, F. B., and Tutin, F., PROC. AM. PHARM. ASSOC., 54 (1906), 352.
(5) Tutin, F., and Clewer, H. W. B., *J. Chem. Soc.*, 95 (1909), 81.
(6) Shinoda, J., and Sato, S., *J. Pharm. Soc. Japan*, 49 (1929), 71; through YEAR BOOK A. PH. A., 18 (1929), 308.
(7) Szent-Györgyi, A., *Nature*, 138 (1936), 27, 798; 139 (1937), 326; 140 (1938), 426.
(8) Fantus, Bernard., Dyniewicz, H. A. and Dyniewicz, J. M., THIS JOURNAL, 22 (1933), 323.
(9) "U. S. Pharmacopœia, XI," Mack Printing Company, Easton, Pa., 1936, p. 473.
(10) McPherson, W., "Elementary Treatise on Qualitative Analysis," 1921 Varsity Supply Co., Columbus, Ohio.
(11) Rosenthaler, L., "The Chemical Investigation of Plants," 1930, p. 22. G. Bell & Sons, Ltd., London.
(12) Power, F. B., and Tutin, F., PROC. AM. PHARM. ASSOC., 54 (1906), 352. Shinoda, J., and Sato, S., *J. Pharm. Soc. Japan*, 49 (1929), 71.

A Pharmacognostical Study of Buchu*

By H. S. Feldman† and H. W. Youngken‡

The National Formulary VI (1) recognizes the leaves of three species of the genus *Barosma* as sources for Buchu, namely: *Barosma betulina* (Thunberg) Bartling et Wendland or "Short Buchu," *Barosma crenulata* (Linné) Hooker or "Oval Buchu" and *Barosma serratifolia* (Curtis) Willdenow or "Long Buchu." The leaves of other species of *Barosma* and also of *Empleurum* have appeared on the drug market as adulterants or substitutes for Buchu.

The chief commercial source for Buchu is the Union of South Africa. *B. betulina* is frequent on the Schurfdebergen, the Olifants River Mountains and the Cedar Mountains, also in the district of Clanwilliam, north of Cape Town and some other parts of the west of Cape Colony. *B. crenulata* has a wider range occurring on the Paarl, Stellenboch, Caledon and Swellendam Mountains. *B. serratifolia* (Fig. 1) was reported from the districts of George and Swellendam to the east of Cape Town (2).

Authentic samples of *B. betulina*, *B. crenulata* and *B. serratifolia* were received from the Union of South Africa through its legation at Washington, D. C. Ten samples of Buchu, N. F. were received from several American sources. All of these samples were used in the histological and chemical studies presented in this paper.

The pharmacognostical studies in this paper were undertaken to improve upon the Buchu monograph in the National Formulary VI (1) and to develop microscopical procedures for distinguishing the leaves of three official species of Buchu from one another.

HISTORY, METHODS AND DESCRIPTION

History.—The historical nomenclature of the plants yielding the three commercial varieties of Buchu leaves has been discussed by Young (3) in 1912. The medicinal properties of Buchu were first discovered by the Hottentots of South Africa. White explorers reported that these people used powdered Buchu leaves to rub on their greased bodies as a perfume. In the native medical practice of these South Africans, Buchu was employed as a brandy in diseases of the stomach, bowels and bladder. Buchu decoctions were prescribed by the Hottentot medicine men as vulneraries for wounds. London's medical profession first became acquainted with Buchu when, in 1821, Reece and Company imported *B. crenulata* from the Cape Colonists of South Africa and R. Reece discussed its usefulness in the *Monthly Gazette of Health* for February 1821.

Throughout the years many writers have considered Buchu as a remedy for various disorders of man. It has long been regarded as a diuretic and diaphoretic, and administered principally in chronic catarrh of the bladder, irritable conditions of the urethra and prostatic affections. In 1880, Bentley and Trimen (4) discussed the three official Buchu leaves and stated that these leaves act as a slight tonic and aromatic stimulant. This drug, according to these authors, was recommended for chronic rheumatism, dyspepsia, dropsy and certain skin

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diseases. Solis-Cohen (5) wrote, in 1928, that Buchu was employeed chiefly as a sedative and antiseptic in infections of the urinary tract, especially in gonorrhea and its complications. Incontinence of urine dependent on irritability of the neck of the bladder is stated to be often relieved by it. Lynn (6) states that Buchu is used as a diuretic, urinary antiseptic and expectorant, principally by the laity and by the older school physicians. In 1931, Potter (7) reported that Buchu was of value in chronic affections of the genito-urinary mucous membranes, on which the volatile oil acts topically, being eliminated by the kidneys and that it was also a useful remedy in pyelitis, cystitis, urethritis and diseases of the prostate gland. He recommended its use in atonic dyspepsia, chronic rheumatism and skin affections. Grieve (8) recorded that Buchu was

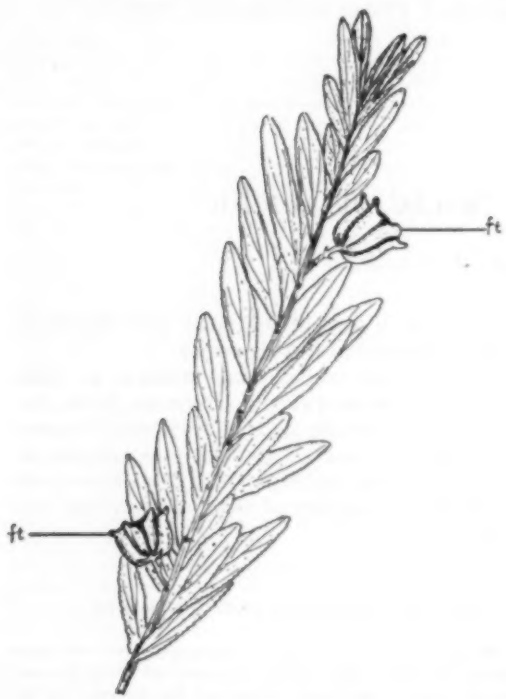


Fig. 1.—Leaf and fruiting branch of *Barosma serratifolia*. *ft*, fruit.

especially useful in gravel inflammation and catarrh of the bladder. In 1932, Watt and Breyer-Brandwijk (9) reviewed the uses of Buchu. They stated that Buchu leaves are a very widely used household medicine in South Africa, usually as a brandy tincture or as a vinegar. These preparations were further stated to have a great reputation in kidney diseases and as a local application to bruises. These writers refer to Pappe who used an infusion of Buchu to stimulate perspiration in rheumatism and gout and the leaf as a remedy in cholera, in catarrh of the bladder, in urinary diseases, in dropsy, as a digestive tonic, and as a bath in chronic rheumatism. Youngken (10) has recorded the uses of Buchu as a disinfectant to the urinary tract in cystitis, pyelitis, urethritis and prostatitis. Sollmann (11) states that Buchu is used as a diuretic in catarrhal cystitis.

Buchu was first mentioned in the second revision of the United States Pharmacopœia of 1840 under the Latin title of Diosma and the English title of Buchu. In the U. S. P. III of 1850, the name Buchu became the official Latin title which persisted in all the subsequent editions of the U. S. P. through the 10th revision. It was dropped from the U. S. P. XI but was introduced into the National Formulary VI and is to be retained in the N. F. VII.

The monograph for Buchu has varied in the U. S. P. revisions in respect to the official species recognized. Table I shows the variations in the official recognized species (U. S. P. III, 1840, to N. F. VII, 1942).

TABLE I.—HISTORICAL RECOGNITION OF OFFICIAL BUCHU[a]

Official Text	Species Recognized
U. S. P. II (1840) (under title of *Diosma*)	*Diosma crenata*
U. S. P. III (1850) (under title of *Buchu*)	*Barosma crenata* and other spp. of *Barosma*
U. S. P. IV (1860)	*Barosma crenata* and other species of *Barosma*
U. S. P. V (1870)	*Barosma crenata* and other species of *Barosma*
U. S. P. VI (1880)	*Barosma betulina*, *B. crenulata* and *B. serratifolia*
U. S. P. VII (1890)	*Barosma betulina* and *B. crenulata*
U. S. P. VIII (1900)	*Barosma betulina*
U. S. P. IX (1910)	*Barosma betulina* and *B. serratifolia*
U. S. P. X (1920)	*Barosma betulina*, *B. crenulata* and *B. serratifolia*
U. S. P. XI (1930)	Not recognized
N. F. VI (1936)	*Barosma betulina*, *B. crenulata* and *B. serratifolia*
N. F. VII (1942)	*Barosma betulina*, *B. crenulata* and *B. serratifolia*

[a]The botanical names are quoted from the official texts

The availability of commercial supplies of varieties of this drug throughout the years may have influenced the United States Pharmacopœial Revision Committee in selecting the species to be included in the Buchu monographs.

The present edition of the Homeopathic Pharmacopœia of the United States of America (12) includes two separate monographs under Buchu. One, "*Barosma Crenata*" or "*Buchu*" and the other "*Barosma Serratifolia*" or "*Buku*." This book does not recognize *B. betulina*. The present British Pharmacopœia (13) recognizes only *B. betulina* as the source of Buchu.

Histological studies on Buchu leaves and stems have been made in the past by a number of investigators. Shimoyama (14), in 1888, reported a microscopical study on several species of Buchu leaves. In 1895, Sieck (15) studied the histologies of various genera of the *Rutaceae* including *Barosma*. Dohme (16), in 1897, reported on the histology and pharmacognosy of Buchu leaves. In 1902, Schulz (17), in a paper, "Anatomy of the Leaves of the *Rutaceae*," recorded a microscopical investigation of Buchu leaves. Solereder (18) in his "Systematic Anatomy," pictured an original drawing of *B. serratifolia* by Willdenow and also discussed the microscopical characters of Buchu leaves. In 1937, Kimura (19) described a study of the structural and histological features of *B. betulina*, *B. crenulata* and *B. serratifolia*. Levin (20), in 1929, described the determi-

nation of the "Vein Islet Number" for Buchu leaves. In 1931, Kimura and Fujikawa (21) tried to distinguish between the leaves of *B. betulina* and *B. crenulata*. They found that, in general, the structural arrangements of the two leaves agree. However, the forms of the stomata constituted a fairly good point of distinction, those of *B. betulina* being the larger. Wallis and Dewar (22) conducted histological studies on Buchu leaves and based some of their results on the differences between the stomata of the varieties. Mansfield (23) had undertaken a study of the microscopy of Buchu stems but recorded only the elements of the powdered stems and their detection in powdered Buchu leaves. He did not discuss the complete histology of the stem of Buchu. Flückiger (24), Planchon (25), Greenish (26), Moll and Janssonius (27), Gilg, Brandt and Schuroff (28), Planchon and Bretin (29) and others have described the anatomical structure of the official Buchu leaves.

TABLE II.—DESCRIPTIVE DIFFERENCES OF THE THREE OFFICIAL BUCHUS

	Barosma betulina	*Barosma crenulata*	*Barosma serratifolia*
Color	Pale olive to a dusky green	Light olive-brown to dusky yellow-green	Moderate olive-green to dusky yellow-green
Shape	Rhomboidally ovate	Oblong-ovate	Linear-lanceolate
Length	5 to 25 mm.	7 to 28 mm.	8 to 33 mm.
Breadth	3 to 17 mm.	3 to 12 mm.	2 to 8 mm.
Apex	Obtuse	Round	Acute
Base	Obtuse	Wedge shaped	Cuneate
Margin	Dentate	Serrate	Sharply serrate
Petiole	1 mm.	2 mm.	1.5 mm.

Methods.—The color determinations of the whole and powdered Buchu leaves are based on the technique given by Judd and Kelley (30).

Transverse sections of the Buchu leaves were cut on a rotary microtome, the material first being imbedded in paraffin. In the process of cutting leaf sections, contact with water was avoided so as to prevent the separation of the epidermis from the palisade region. This separation, if it occurs, is caused by the swelling of the mucilage-containing inner walls of the epidermal cells upon contact with water, and this forms the pseudo-hypodermis. Petiole sections were prepared from materials which had been placed between the split halves of a cork stopper. Stem transverse sections were cut from paraffin-imbedded material. Fresh stems, dehydrated with the Butyl Alcohol series, were set in paraffin blocks. Sections five microns in thickness were cut from these paraffin blocks on the rotary microtome. Safranin (1 Gm. in 100 cc. of 95% alcohol) diluted with water was used as the primary stain in the process of staining. The counterstain employed was Fast Green (1 Gm. in 25 cc. of 100% alcohol plus 75 cc. of Oil of Cloves) diluted with 95% alcohol. Samples of powdered Buchu leaves and stems were mounted in the following reagents for various microscopical tests: Phloroglucin-HCl; 5% solution of Potassium Hydroxide; Chloral Hydrate Solution; Iodine Water; Schulze's Maceration Fluid; 70% Alcohol; 95% Alcohol; 100% Alcohol; Xylol; Petroleum Ether.

"Vein Islet Number" determinations were based on a method given by Zufall and Burlage (31). Two pieces of leaf material are obtained and examined as follows: Each end of the leaf is cut off at a point one-fourth of the distance from tip to base and discarded, the piece left is cut down the mid-rib and from each side a piece is obtained by cutting away one-fourth of the outer edge and one-fourth next to the mid-rib. These areas are used because in them the vein islets are found to be uniform in size. The vein islets next to the mid-rib are larger than in the other regions, while in the margins of some leaves no vein islets are found. After clearing with chloral hydrate solution or solution of sodium hypochlorite, the sections are mounted on glass slides in water and examined under the microscope. The area of the vein islet may be determined by counting the number of vein islets within the field of the 16-mm. objective. The number of vein islets in the field divided by the area of the field (in millimeters) gives the average area of the vein islet. The 16-mm. objective showed a field whose area was 1.98 sq. mm. This is near the whole number, 2, so that the "Vein Islet Number" can be easily calculated. The vein islets in a field are counted, and the "Vein Islet Number" is obtained by dividing this figure by 2.

For the palisade ratio determinations, the leaves are cut into pieces 3 mm. square and placed in a test tube containing 10 cc. of chloralphenol (equal parts by weight of chloral hydrate and phenol). The tube is heated on a water bath for twenty minutes and then the leaf material is removed and mounted in a few drops of the reagent. Mounts of the powdered leaves are prepared in a similar manner for microscopical examination. The palisade ratio is obtained by counting the total number of palisade cells beneath four epidermal cells and dividing the total number by four.

A number of official leaves were examined by the following method for stomatal measurements: The lower epidermis is removed by macerating the leaves in 5% KOH solution for five minutes on a water bath. Fragments of this material are mounted in dilute glycerin and examined. Powdered leaf mounts are prepared in a like manner. The measurements are made with a standardized microscope, the low and high power objectives having values of 17.4 and 3.8 microns per ocular micrometer scale division, respectively.

Samples of the official Buchu leaves were examined for volatile oil content by the Clevenger method (32). Total ash and acid-insoluble ash determinations were carried out on 12 samples of Buchu leaves according to the U. S. P. XI method (33).

Description.—*B. betulina* leaves (Fig. 2) are rhomboidal to ovate to rhomboidally obovate, from 5 to 25 mm. in length and from 3 to 17 mm. in breadth; apex obtuse and sometimes recurved; base obtuse or wedge-shaped; margin dentate and glandular-punctate with an oil gland at the base of each marginal tooth; upper surface papillose; lower surface longitudinally striate; texture coriaceous; petioles up to 1 mm. in length; color pale olive to a dusky yellow-green. (Table II.)

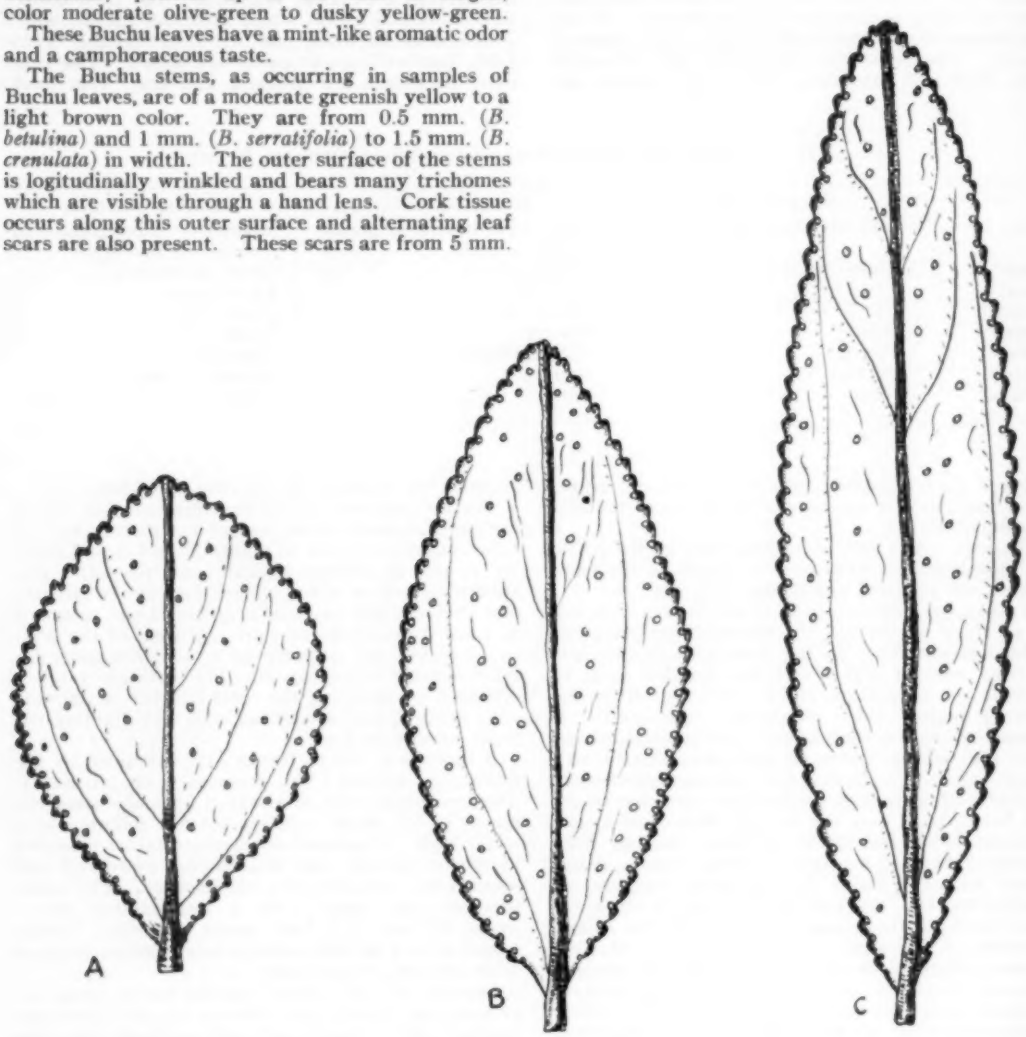
The leaves of *B. crenulata* (Fig. 2) are oblong ovate, from 7 to 28 mm. in length and from 3 to 12

mm. in breadth; apex rounded and occasionally recurved; base obtuse or wedge-shaped; margin serrate and glandular-punctate with an oil gland at the base of each tooth; surface papillose above, logitudinally striate beneath; brittle and coriaceous; petiole up to 2 mm. in length; color light olive-brown to dusky yellow-green.

B. serratifolia leaves (Fig. 2) are lanceolate, from 8 to 33 mm. in length and 2 to 8 mm. in breadth; apex acute; base cuneate; margin sharply serrate with oil glands at the marginal teeth bases; upper surface punctate; lower surface striated; texture coriaceous; petioles up to 1.5 mm. in length; color moderate olive-green to dusky yellow-green.

These Buchu leaves have a mint-like aromatic odor and a camphoraceous taste.

The Buchu stems, as occurring in samples of Buchu leaves, are of a moderate greenish yellow to a light brown color. They are from 0.5 mm. (*B. betulina*) and 1 mm. (*B. serratifolia*) to 1.5 mm. (*B. crenulata*) in width. The outer surface of the stems is logitudinally wrinkled and bears many trichomes which are visible through a hand lens. Cork tissue occurs along this outer surface and alternating leaf scars are also present. These scars are from 5 mm. (*B. betulina* and *B. crenulata*) to 10 mm. (*B. serratifolia*) apart. The fracture of the stems is short and slightly fibrous. The odor is aromatic; the taste, bitter and aromatic.



Percentage of Stems in Buchu, N. F.—The National Formulary VI (1) permits up to 8% of stems in Buchu. Samples of Buchu were examined and the percentages of stems determined. The results are expressed in the Table III.

HISTOLOGY

Histology of Barosma betulina Leaf.—In transverse view the leaf of *B. betulina* show as thick uneven and striated cuticle on the upper and lower epidermises. Below the cuticle of the upper epidermis lies a single row of large tangentially elongated epidermal cells containing many sphaero- and feather-crystals of hesperidin. The inner walls of these epidermal cells are mucilaginous, and when water contacts them they swell and produce a mucilaginous pseudo-hypodermis. In some of the permanent sections examined a little separation had occurred due to the small amount of water present in the 70% alcohol which had been used as a preservative for some of the fresh leaves. Other sections prepared from fresh material preserved in dehydrated alcohol did not show any pseudo-hypodermis. This pseudo-hypodermis has been referred to in the literature as the sub-epidermis. Beneath the region of the pseudo-hypodermis is a single row of palisade cells that borders loose subjacent spongy parenchyma tissue. The palisade cells contain numerous chloroplastids. The loose spongy parenchyma forms chains of cells which surround numerous air spaces. These parenchyma cells contain rosettes of calcium oxalate (19 microns), chloroplastids and

hesperidin crystals. Isolated groups of fibers are found among the spongy parenchyma tissue. Circular oil cavities bounded by secretory epithelium occur in the mesophyll either half way between the mid-rib and the end of the leaf or near the margin of the leaf. Vascular tissue courses through the intraneural leaf region. This tissue, when seen in radial view, show spiral tracheae. The mid-rib contains a large meristele of fibrovascular bundles which have a broad xylem and a narrow phloem. The bundles are separated by rays one cell in width. Beneath the phloem occurs a crescent-shaped arc of nonlignified sclerenchyma fibers, and below this arc the mesophyll cells, abundant in hesperidin crystals. The lower epidermis is wavy, its cells are smaller than those of the upper and also contain hesperidin. Stomatal pores interrupt the lower epidermal cuticle which is more striated than the upper cuticle. In surface view (Fig. 3 (*A*)), the upper epidermis discloses large polygonal-shaped cells with straight vertical walls, nonstriated outer walls and hesperidin content. The lower epidermis, in surface section [Fig. 3 (*B*)], shows many broad, elliptical stomata up to 52.2 microns in length and surrounded by 4 to 6 neighboring cells. The lower epidermal cells are smaller in size than those of the upper and also contain hesperidin.

TABLE III

Sample No.	American	Sample No.	Union of South Africa
1	2%	10	1.5%
2	3%	11	1%
3	3.5%	12	1%
4	8%	13	7%
5	7.5%	14	7%
6	8%	15	7.5%
7	7%	16	7%
8	6.5%	17	6%
9	7%	18	6%

Histology of Barosma crenulata Leaf.—The leaf of *B. crenulata* (Fig. 4) in transverse view exhibits a layer of large, sausage-shaped epidermal cells covered by a thick, wavy and striated cuticle. The epidermal cells contain many hesperidin feather-crystals (Fig. 4 (*h*)) and possess mucilaginous inner walls which lay down a pseudo-hypodermis when in contact with water. A layer of palisade parenchyma containing chloroplastids is underneath, which is followed by a region of loose mesophyll tissue. The rosettes found in the mesophyll measure up to 26.6 microns. The intraneural leaf region shows vascular tissue of the large veins, circular oil secretion cavities and a few groups of nonlignified fibers. The tracheae of the vascular tissue in radial view show spiral aud scalariform markings. The mid-rib contains a large meristele of fibrovascular bundles having a broad upper xylem and a narrow lower phloem, beneath which occur several rows of nonlignified sclerenchyma fibers. Vascular rays, one cell in width, separate the bundles; the secondary phloem shows the development of hard bast. The lower epidermis consists of a single row of narrow, bullate epidermal cells with hesperidin sphaero-crystals in many of the cells. This epidermis is covered by a very thick and striated cuticle which is interrupted by stomatal pores or openings. The upper epidermis, in surface view, exhibits large polygonal cells with slightly beaded, vertical walls, smooth outer walls and abundant hesperidin crystals. The lower epidermis shows smaller polygonal epidermal cells with hesperidin content. The stomata on this epidermis are up to 43.5 microns in length and are surrounded by as many as 4 neighboring cells.

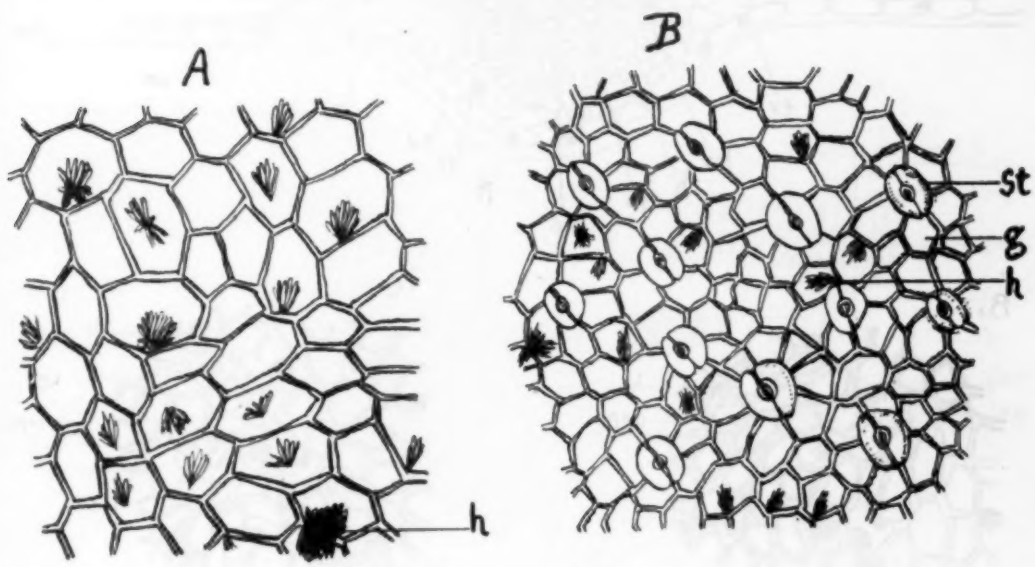


Fig. 3.—*Barosma betulina.*

A—Surface view of upper epidermis of lamina: *h*, hesperidin crystals. *B*—surface view of lower epidermis of lamina: *st*, stomata; *g*, neighboring cell; *h*, hesperidin crystals.

Histology of Barosma serratifolia Leaf—This leaf (Fig. 5), in transverse view, has the following anatomical features: a wavy, striated, thick cuticle covering a layer of rounded to tangentially elongated upper epidermal cells which contain hesperidin, the inner walls of these cells, as in the case of *B. betulina* and *B. crenulata*, form a pseudo-hypodermis of mucilage, when water is added to the section.

Below this layer is a single row of palisade cells containing chloroplastids. Loose mesophyll cells with rosettes of calcium oxalate up to 22.8 microns in diameter lie beneath the palisade region. Circular oil cavities with secretory epithelium occur in the mesophyll, chiefly near the margin of the leaf. The mid-rib of the leaf contains a large meristele of bundles having a broad xylem and a narrow phloem separated from each other by medullary rays one cell in width. An arc of several rows of nonlignified pericyclic fibers are below the phloem tissue. The bundles of several large veins course through the mesophyll and show only spiral-marked traceae in radial view. The lower epidermis consists of more or less elongated cells smaller in size than the upper epidermal cells. They also contain feather- and sphaero-crystals of hesperidin. A thick cuticle, which is perforated in places in stomatal pores, covers the lower epidermis. In surface sections (Fig. 5 (*B*)), the upper epidermis shows polygonal cells with slightly beaded inner walls and slightly striated outer walls. Hesperidin crystals occur in both these cells and those of the lower epidermis. The lower epidermis (Fig. 5 (*C*)) in surface view exhibits many stomata with 3 to 5 neighboring cells. The stomata are up to 38.2 microns in length. (Table IV.)

Histology of Buchu Stems.—Transverse and radial longitudinal sections were made from the stems of the official species and used for histological studies. The histologies of the three stems were found to be similar with the exception of the pith region of *B. betulina*. This region is smaller than the corresponding regions of *B. crenulata* and *B. serratifolia*. Also, slight differences in the stems were discovered in the sizes of the calcium oxalate rosettes, trichomes and the number of oil secretion reservoirs in the cortex. The stem histology of *B. betulina* is typical for the 3 Buchu species. In transverse view (Fig. 6), this stem presents a single layer of large irregular epidermal cells from which arise short, papilla-like trichomes and unicellular, nonglandular hairs. These hairs measure up to 119 microns in length and 20.4 microns in width. (*B. crenulata* has hairs up to 102 microns long and 15.6 microns wide; the hairs of *B. serratifolia* are up to 108 microns in length and 17 microns in width; the bases of these hairs as compared with those of *B. betulina* and *B. crenulata* are more bulbous.) Some of the epidermal cells contain

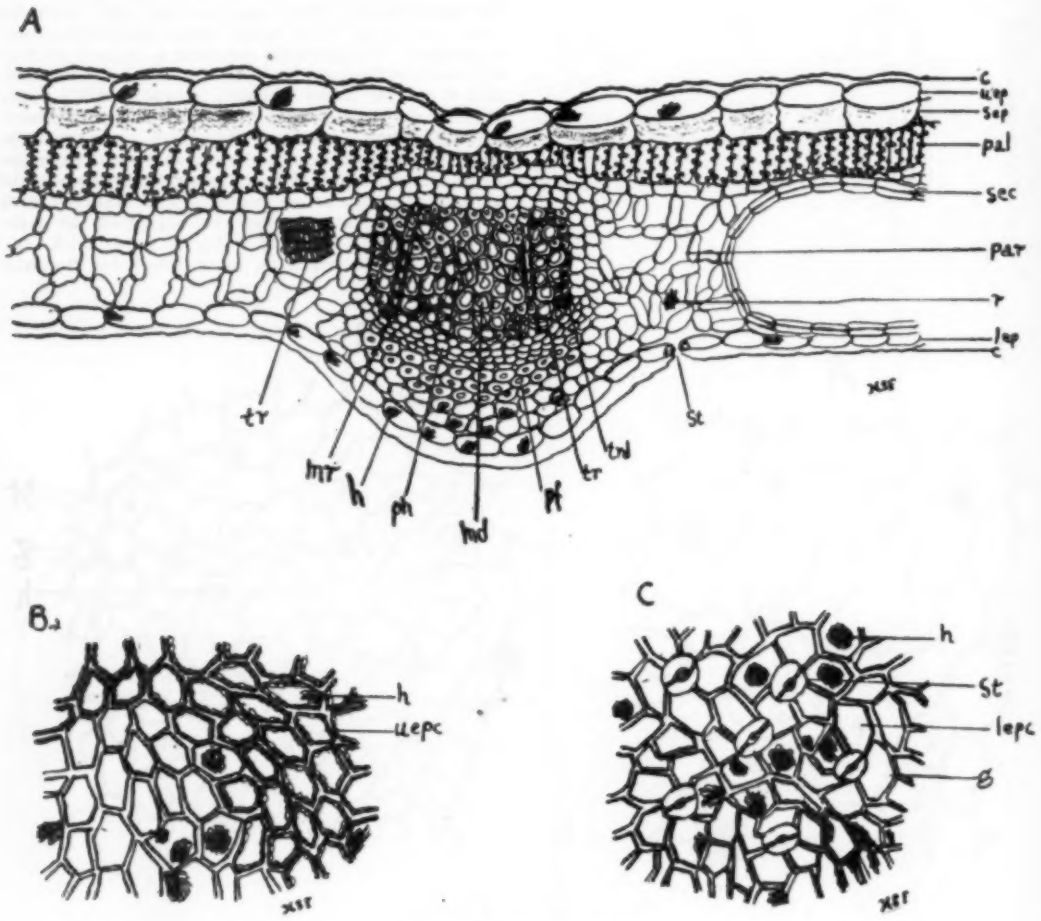


Fig. 4.—*Barosma crenulata*.

A—Transverse section of the Leaf blade: *c*, cuticle; *uep*, upper epidermis; *sep*, pseudohypodermis or sub-epidermis; *pal*, palisade; *sec*, secretion sac; *par*, spongy parenchyma; *r*, rosette (CaC_2O_4); *lep*, lower epidermis; *st*, stoma; *trd*, tracheid; *tr*, trachea; *pf*, pericyclic fibers; *md*, mid-rib; *ph*, phloem; *h*, hesperidin; *mr*, medullary ray. *B*—upper epidermis in surface view: *h*, hesperidin; *uepc*, upper epidermal cell. *C*—lower epidermis in surface view: *st*, stomata; *lepc*, lower epidermal cell.

TABLE IV.—MAIN HISTOLOGICAL DIFFERENCES BETWEEN THE THREE OFFICIAL BUCHU LEAVES

Anatomical Features	*Barosma betulina*	*Barosma crenulata*	*Barosma serratifolia*
Inner walls of upper epidermis	Straight	Slightly beaded	Slightly beaded
Outer walls of upper epidermis	Smooth	Smooth	Slightly striated
Tracheae of vein bundles	Spiral	Spiral and scalariform	Spiral
Location of oil cavities	Halfway between mid-rib and margin	Chiefly intraneural regions	Near margin
Rosettes of calcium oxalate	Up to 19 microns	Up to 26.6 microns	Up to 22.8 microns
Size of stomata (length)	52.2 microns	43.5 microns	38.2 microns
Number of neighboring cells	4 to 6	4 to 5	3 to 5

a brownish red resinous substance, while others possess hesperidin crystals. The epidermal layer is covered by a thick cuticle; and the inner walls of the epidermal cells, as in the leaf, are mucilaginous and produce a pseudo-hypodermis when water is present. A narrow cortex lies beneath the pseudo-hypodermal region. The cortical cells are comparatively larger than the epidermal cells and also contain hesperidin crystals. A single secretion reservoir often occurs in this region of the stem (*B. crenulata* has up to 3 reservoirs, while *B. serratifolia* possesses 4). Beneath the cortex lies a pericycle consisting of an interrupted circle of slightly lignified fibers. The phloem tissue below the pericycle is narrow and is divided into numerous patches by phloem rays which are one cell in width. The xylem region is broad and is likewise divided into radiating xylem wedges by xylem rays one cell in width. The pith region is central and consists of large parenchyma cells containing rosettes of calcium oxalate up to 19 microns

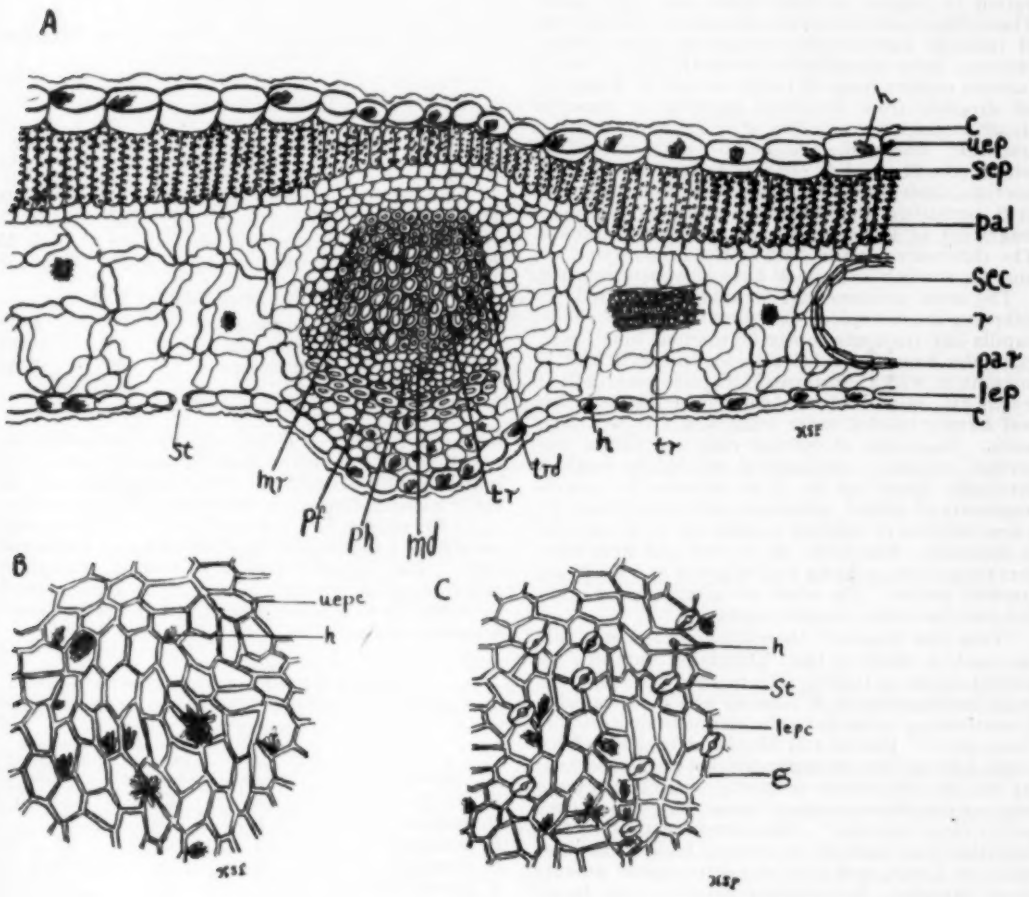


Fig. 5.—*Barosma serratifolia*.

A—Transverse section of the leaf blade: *c*, cuticle; *uep*, upper epidermis; *pal*, palisade; *sep*, sub-epidermis, *h*, hesperidin; *scc*, secretory reservoir; *r*, rosette of calcium oxalate; *par*, parenchyma; *lep*, lower epidermis; *tr*, trachea; *trd*, tracheid; *md*, mid-rib region; *ph*, phloem; *pf*, pericyclic fibers; *mr*, medullary ray; *st*, stomata. *B*—upper epidermis in surface view: *uepc*, upper epidermal cell; *h*, hesperidin. *C*—lower epidermis in surface view: *st*, stomata; *lepc*, lower epidermal cell.

(*B. crenulata* has rosettes up to 11.4 microns, and in *B. serratifolia*, the rosettes measure up to 15.2 microns). This region is smaller in *B. betulina* than in either *B. crenulata* or *B. serratifolia*.

Histology of the Petioles.—Sections were made from the petioles of the 3 Buchu species and examined. No histological distinctions between the 3 petioles are evident. In transverse view, all show a very thick cuticle covering large rectangular to square-shaped epidermal cells from which arise a few short unicellular, nonglandular hairs. A broad cortex consisting of rectangular parenchyma cells with hesperidin content lies beneath the epidermis. One to three secretion reservoirs are present in the cortex. A large fibrovascular bundle lies in the center of the petiole. The bundle has a broad xylem area through which single-celled xylem rays extend. A narrow phloem with one-celled phloem rays is present beneath the xylem region.

Histology of Powdered Buchu.—Powders ground to No. 60 mesh were prepared from each stem and leaf of the 3 species, and these were examined separately. Scrapings were made from the stems to observe the types and sizes of trichomes. Powdered leaves of *B. betulina*, *B. crenulata* and *B. serratifolia* offered the following histological elements: Fragments of epidermal cells with thick cuticle and containing sphaero- and feather-crystals of hesperidin or diosmin (in surface view, these epidermal cells varied in respect to their inner and outer walls. These differences have been recorded.) Fragments of palisade parenchyma containing many chloroplastics; loose mesophyll tissue with few rosettes of calcium oxalate from 15 to 30 microns in diameter; oil droplets from secretion reservoirs; vascular tissue fragments consisting of spiral and reticulate tracheae; nonlignified fibers up to 500 microns in length (the fibers of *B. betulina* measured up to 500 microns, those of *B. crenulata* up to 295 microns and in *B. serratifolia* the fibers were up to 350 microns); fragments of lower epidermal cells with stomata. The differences in the sizes of the stomata and the number of neighboring cells have also been recorded.

The stem powders and scrapings presented the following microscopical structures: Numerous short papilla-like trichomes up to 65 microns long; nonglandular hairs up to 119 microns in length and 17 microns in width; brownish, angular cork cells in fragments; large epidermal cells covered by a cuticle and having beaded inner walls and striated outer walls; fragments of cortical cells containing hesperidin crystals; nonlignified to slightly lignified pericyclic fibers up to 1130 microns in length; fragments of pitted, reticulate and spiral tracheae; a few rosettes of calcium oxalate up to 19 microns in diameter. The colors of the leaf and stem powders range from a dusky yellow-green to a moderate greenish yellow. The odors are aromatic and mint-like and the tastes camphoraceous.

"Vein Islet Number" Determinations.—Levin (34) has made a study of the "Vein Islet Numbers" of several species of Buchu. His method was employed in an investigation of *B. betulina* and *B. serratifolia*. A controversy arose as to the complete definition of "vein islet." Eames and MacDaniels (35) defined a vein islet as "the ultimate division of the conducting strands completely or partly encircling minute areas of the photosynthetic tissue with which they are in close contact." The interpretation of this definition was applied to camera lucida drawings made by Levin, and also to microscopical mounts of our samples. A conclusive determination based on this definition could not be reached in finding the vein islet numbers. Benedict (36) has given the name "vein islet" to the small area of the leaf enclosed by the smallest branching veins. This term also led to indefinite results. Benedict's work was reviewed by Ensign (37), and in his second paper (38) Ensign states, "During the progress of this work, certain questions arose regarding the accuracy of the methods employed by Benedict in determining the area of vein islets in the leaves which he used." For practical results we employed a modification of the Eames and MacDaniels (35) definition to obtain the final vein islet number for the leaves examined. The modification omitted the words "partly encircle"; the definition as used was "the ultimate divisions of the conducting strands completely encircling minute areas, etc." The term "Vein Islet Number" was coined by Levin (34) who defined it as "the number of vein islets in a square millimeter of leaf tissue." His method of determining these numbers was as follows:

First, the leaves were cleared by using solutions of chlorinated soda and chloral hydrate. Then, at a definite magnification, the image of the leaf was projected, by means of a series of prisms, on to a horizontally placed sheet of paper upon which a rectangular area representing four square millimeters of leaf tissue had been traced. The islets which fell within this area were counted, and from this figure the vein islet number was obtained by dividing the gross number of islets by four. (Table V.)

Thirty leaves, ten of each of the three official species of *Barosma*, were examined (see *Methods*) and their vein islet numbers determined.

TABLE V.—COMPARISON OF VEIN ISLET NUMBER DETERMINATIONS

Species	Method of Examination	No. Leaves Examined	Average V. I. N.
Levin's Determination of V. I. N.			
B. betulina	Camera lucida	7	11.28
B. betulina	Projection	27	12.77
B. serratifolia	Camera lucida	4	19.85
B. serratifolia	Projection	21	20.58
Microscopical Determination of V. I. N.			
B. betulina	Microscope	10	8.43
B. crenulata	Microscope	10	10.65
B. serratifolia	Microscope	10	16.62

Palisade Ratios of Official Buchu Leaves.—Zornig and Weiss (39) reported that the average number of palisade cells beneath an upper epidermal cell affords a useful diagnostic character. Wallis and Dewar (40) defined the palisade ratio as the "average number of palisade cells beneath an upper epidermal cell." The palisade ratio of *B. betulina* was given as a range from 10 to 26. Our findings are expressed in Table VI as a single figure so as to insure more accurate results.

TABLE VI.—PALISADE RATIOS

Species	No. of Leaves Examined	No. of Determinations	Average Palisade Ratio
B. betulina	10	25	25
B. betulina	8	30	26
B. crenulata	12	25	24
B. crenulata	10	20	23.2
B. serratifolia	10	25	20
B. serratifolia	12	20	22.7

Stomatal Measurements.—Wallis and Dewar (41) have measured the stomata of the official Buchu

leaves and a number of other species of *Barosma*. They found that the dimensions of the stomata have proved of value in distinguishing the official leaves from the leaves of all the other species of *Barosma* examined. Kimura and Fujikawa (21) found that the stomata of *B. betulina* were larger than those of *B. crenulata*. Wallis and Dewar (41) found stomata of *B. betulina* measuring up to 51.6 microns. They did not measure the stomata of *B. crenulata* or of *B. serratifolia*. (Table VIII).

respectively. A report by Schimmel and Co. (45) gave the yield of oil from *B. crenulata* as 1.7%. The average yields recorded by Gildemeister (46) were 1.3% to 2.5% for *B. betulina*, 1.7% for *B. crenulata* and 0.8% to 1% for *B. serratifolia*. Parry (47) states that the yield of oil from the 3 official Buchu species is from 1% to 2%. The American Pharmaceutical Association Laboratory (48) has investigated the volatile oil content of *B. betulina* and *B. crenulata* and found that they yielded 1.9% and 2.2%, respectively.

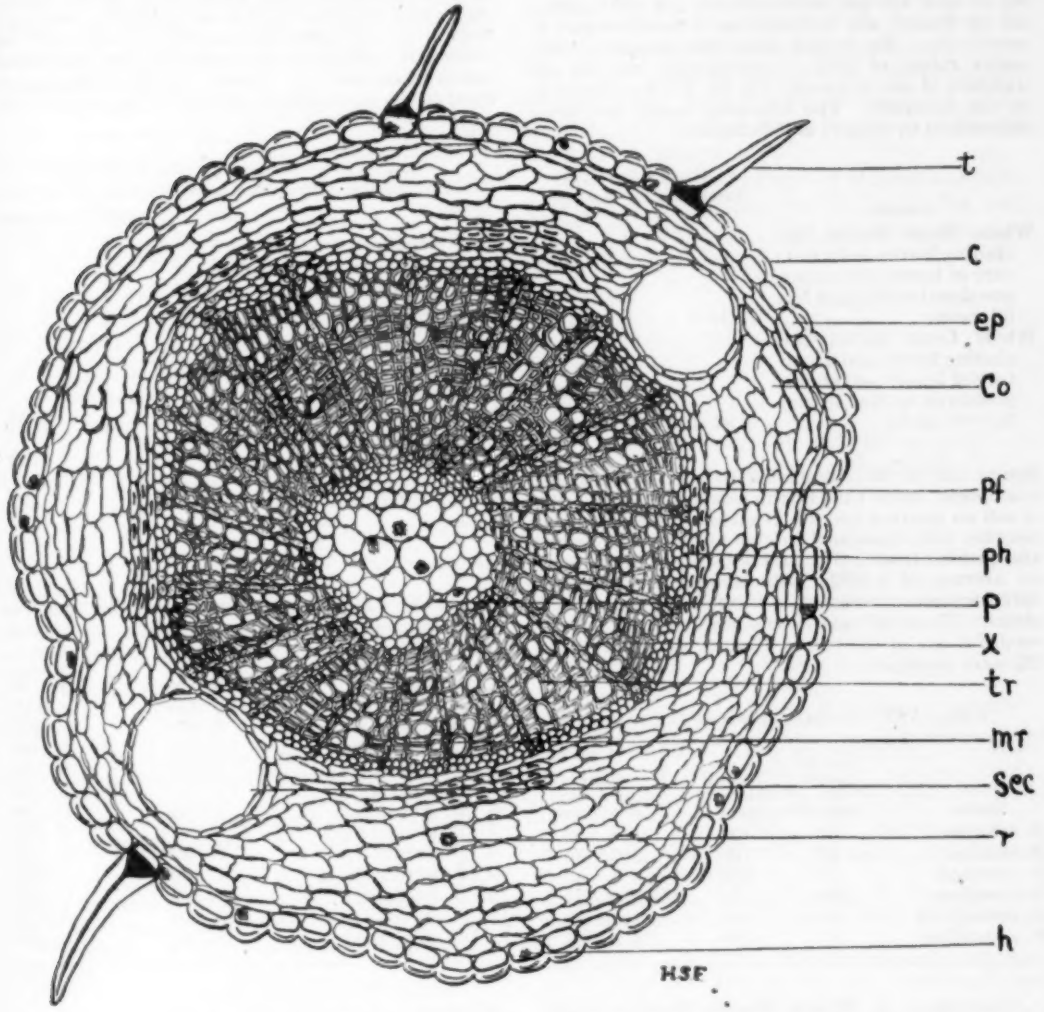


Fig. 6.—Stem of Short Buchu, *Barosma betulina*, in transverse section.

c, cuticle; *ep*, epidermis; *co*, cortex; *pf*, pericyclic fibers; *ph*, phloem; *x*, xylem; *tr*, trachea; *mr*, medullary ray; *sec*, oil-secretion reservoir; *r*, rosette aggregate crystal of calcium oxalate; *h*, hesperidin crystals; *p*, pith; *t*, unicellular, non-glandular trichome.

Volatile Oil of Buchu.—Oil of Buchu has been distilled from the leaves of several species of *Barosma* The three official Buchu leaves are recognized as major commercial sources for this oil. Brandes (42) first prepared the volatile of Buchu in 1827. He reported 0.88% as the average yield. Bedford (43) experimented with *B. crenulata* and *B. serratifolia*. He stated that the former yielded more oil than the latter, the yields being 1.21% for the Oval Buchu and 0.66% for the Long Buchu. In 1890, Thompson (44) recorded the percentages of oil in *B. betulina*, *B. crenulata* and *B. serratifolia* as 1.45, 1.6 and 1, Table VIII gives the results obtained by our investigation.

The constituents of the volatile oil of Buchu have been determined by Kondakow (49) and Bialobrzeski (50). These ingredients are diosphenol, a crystalline solid, the terpenes limonene and dipentene, and a ketone called laevo-monthone. Semmler and McKenzie (51) stated that the activity of Buchu leaves was due to a camphoraceous substance called "ketophenol" which is present in considerable quantities in the volatile oil of Buchu. An assay for this ketophenol has been described by de Waal (52). Oil of

Buchu is slightly antiseptic and has been used somewhat in medicine.

Total and Acid-insoluble Ash.—In 1879, Jones (53) reported on the ash and soluble matter in three kinds of Buchu. His total ash results are as follows:

B. betulina.....4.69%; 4.47%; 4.40%
B. crenulata.....4.32%; 4.01%; 5.39%
B. serratifolia.....5.03%; 5.55%; 5.22%

Newcomb (54) discussed the results of a large number of total ash and acid-insoluble ash tests carried out by Rogers and Folkstad on *B. betulina* and *B. serratifolia*. He stated that the present (1921) purity rubric of 10% is satisfactory but the ash standard of not to exceed 4% for Buchu seemed to be too stringent. The following results are those determined by Rogers and Folkstad:

Sample	Total Ash, %	Acid-insoluble Ash, %
Whole Short Buchu (including leaves and a mixture of leaves and stems, powdered to No. and No. 60 mesh)	3.34–5.1	0.16–1.39
Whole Long Buchu (including leaves and a mixture of leaves and stems, powdered to No. 40 and No. 60 mesh)	3.84–4.57	0.21–1.49

Squire (55) in his companion to the British Pharmacopoeia, states that Buchu leaves yield about 5% of ash on ignition and 6% is seldom exceeded. Ten samples were examined in this author's laboratory and yielded from 3.6% to 5.68% of total ash with an average of 4.52%. All samples used for our determinations consisted of mixtures of leaves and stems. These mixtures were powdered to No. 60 mesh for use in the tests. Results shown in Table IX were obtained.

TABLE VII.—STOMATAL MEASUREMENTS

Species	No. of Leaves Examined	No. of Measurements	Range of Size in Microns
B. betulina	12	100	34.8 –52.2
B. betulina	10	100	33.55–53.5
B. crenulata	10	100	26.10–43.5
B. crenulata	10	100	26.10–34.8
B. serratifolia	12	100	31.30–38.2
B. serratifolia	10	100	24.30–38.2

Adulteration of Official Buchu Leaves.—Many adulterants for official Buchu have appeared on the drug market. Sage (56) reported that a parcel of Buchu called "Aniseed-Buchu" occurred on the London market. The drug had the appearance of Karoo Buchu (derived from *Diosma succulenta* L.), which was previously described by Sage. However, these leaves had a different odor resembling that of anise and cowslip flowers. It was suggested that this drug had been derived from *Agathosma varabilis*. Holmes (57) discovered that the leaflets of *Psoralea obliqua*, a leguminous plant, had been used to adulterate *B. betulina*. *Barosma venusta* E. and Z. was first exhibited on the London market in 1911. *B. venusta* is a shrubby plant, attains over a foot in height and bears crowded rodlike branches with slender twigs. The leaves on the upper part of the branches are broadly ovate or rounded, being 1/4 to 1/4 inch long and almost as broad. The lower ones may attain 1/2 inch in length and are narrower. The leaves are erect and shortly stalked, bearing impressed marginal glands and smaller glands dotted throughout the soft tissues. The plant is distinguished from that of *Barosma pulchella* (L.) Bartl. and Wendl. by the rounded shape of the leaves and the absence of a citronella odor. Mansfield (23) has described a species of Buchu stems as an adulterant for Buchu. Dummer (58) described *Barosma Peglerae* as a new South African species of Buchu. In 1912, Mansfield (59) found Klip Buchu leaves, *Adenandra fragrans*, used as an adulterant for Long Buchu, on the American market. Jensen (60) described *B. venusta* as an adulterant for the Buchu leaves in current use. Some samples of Buchu offered to the American trade have been found by the officials of the U. S. Dept. of Agriculture (61) to be non-official (1917). The so-called Long Buchu consisted of leaves of *Empleurum serrulatum* Ait., and that sold as Short Buchu proved to be *B. crenulata* var. *latifolia*. The flavor of all but the last of these was distinctly different from the official Buchu leaves. Watt and Breyer-Brandwijk (62) have reported that the leaf of *Barosma Eckloniana* Bartl. has been marketed for Buchu. This leaf is shorter, wider, and more nearly circular than the leaf *B. betulina*.

TABLE VIII.—VOLATILE OIL OF BUCHU

Sample	Yield, %	Average Yield, %
1 (*B. betulina*)	2.1	
1*a* (*B. betulina*)	1.8	1.95
2 (*B. crenulata*)	1.8	
2*a* (*B. crenulata*)	1.5	1.66
3 (*B. serratifolia*)	0.89	
3*a* (*B. serratifolia*)	0.82	0.85
4 (*B. betulina*)	2.0	
4*a* (*B. betulina*)	1.9	1.95
5 (*B. crenulata*)	1.7	
5*a* (*B. crenulata*)	1.8	1.73
6 (*B. serratifolia*)	0.81	
6*a* (*B. serratifolia*)	0.73	0.77

TABLE IX.—TOTAL ASH AND ACID-INSOLUBLE ASH

Sample	Total Ash, %	Acid-Insoluble Ash, %
1 (*B. betulina*)	4.328	0.147
2 (*B. betulina*)	5.893	0.063
3 (*B. betulina*)	4.704	0.080
4 (*B. crenulata*)	4.851	0.136
5 (*B. crenulata*)	5.137	0.194
6 (*B. crenulata*)	4.982	0.128
7 (*B. serratifolia*)	4.603	0.073
8 (*B. serratifolia*)	5.540	0.153
9 (*B. serratifolia*)	4.430	0.009
10 (*B. betulina*)	4.372	0.065
11 (*B. crenulata*)	4.910	0.124
12 (*B. crenulata*)	5.740	0.199

SUMMARY

1. A brief history of the medicinal use and official recognition of Buchu is given.

2. The methods used in the various

pharmacognostical studies are described..

3. Descriptions of the official Buchu leaves and stems are presented.

4. Histological studies have been made on Buchu leaves and stems for a means of distinguishing between the three species. These studies include the following: transverse sections of *B. betulina*, *B. crenulata*, *B. seratifolia*, leaves, stems and petioles; histology of the powdered drug including leaves and stems; Vein Islet Number determinations; palisade ratios; stomatal measurements.

5. Surface sections of both the upper and lower epidermis of the three leaves are discussed. The outstanding differences are found in the radial and outer walls of the upper epidermis; *viz.*, the inner or radial walls are straight for *B. betulina* and slightly beaded for both *B. crenulata* and *B. serratifolia* the outer walls are smooth for *B. betulina* and *B. crenulata* but are slightly striated for *B. serratifolia*.

6. The main diagnostic histological landmarks useful in distinguishing between the Buchu laminae and stems are the short papilla-like trichomes and longer nonglandular hairs. These hairs are found only on the stems and petioles. No hairs were observed on any of the leaf blades.

7. The drug powders of Buchu leaf and stem are described. Some of the main differences are found in the measurements of fibers and calcium oxalate rosettes.

8. The volatile oil of Buchu is discussed as to the percentage yield from each species, its constituents and properties.

9. The results of the determinations of the volatile oil contents of *B. betulina*, *B. crenulata* and *B. serratifolia*, by means of the Clevenger method are given. Average determinations were 1.95% of oil from Short Buchu, 1.13% from Oval Buchu and 0.81% from Long Buchu.

10. The purity rubric of Buchu has been determined according to U. S. P. XI and N. F. VI methods. The following average results were obtained:

	Stems, %	Other Foreign Organic Matter, %	Acid-Insoluble Ash, %
B. betulina	2.6	1	0.089
B. crenulata	7.8	1.5	0.164
B. serratifolia	6.8	0.9	0.078

11. Several common and rare adulterants for official Buchu leaves are discussed.

REFERENCES

(1) "National Formulary VI," Mack Printing Co., Easton, Pa., 1936, p. 61.

(2) Marloth, R., "The Flora of South Africa," Darter Bros. and Co., Capetown, 2 (1935), 109.

(3) Young, W. Y., *Am. J. Pharm.*, 84 (1912), 259.

(4) Bentley, R., and Trimen, H., "Medicinal Plants," J. and A. Churchill, London, I 1880, under Pl. 45, 46, 47.

(5) Solis-Cohen, S., and Githens, T. S., "Pharmactotherapeutics," D. Appleton & Co., New York, 1928, p. 839.

(6) Lynn, E. V., "Pharmaceutical Therapeutics," Ed. 1, McGraw-Hill Book Co., New York, 1929, p. 198.

(7) Potter, S. O. L., "Therapeutics, Materia Medica & Pharmacy," Ed. 15, P. Blakiston's Son & Co., Philadelphia, 1931, p. 195.

(8) Grieve, M. A., "A Modern Herbal," J. Cape, London, 1931, Vol. I, p. 134.

(9) Watt, J. W., and Breyer-Brandwijk, M. G., "The Medicinal and Poisonous Plants of Southern Africa", E. and S. Livingstone, Edinburgh, 1932, p. 89.

(10) Youngken, H. W., "A Textbook of Pharmacognosy," ed. 4, The Blakiston Co., Philadelphia, 1936, p. 447.

(11) Sollmann, T., "A Manual of Pharmacology," Ed. 6, W. B. Saunders Co., Philadelphia, 1942, p. 167.

(12) "Homeopathic Pharmacopœia of the United States," Ed. 5, 1938, pp. 150–151.

(13) "British Pharmacopœia," 1932, p. 93.

(14) Shimoyama, Y., *Arch. Pharm.*, 226 (1888), 64.

(15) Sieck, "Schizolysigen Sekretbehälter," Diss. Bern, 1845, 46 pp.

(16) Dohme, A. R. L., *Drug. Circ. and Chem. Gaz.* 41 (1897), 100.

(17) Schulz, H., "Beitrage z. Blattanatomie d. Rutaceen," Diss. Heidelberg, 1902, 50 pp.

(18) Solereder, H., "Systematic Anatomy of The Dicotyledons," Clarendon Press, Oxford, 1908, Vol. I, p. 175.

(19) Kimura, K., *J. Pharm. Soc. Japan;* through *Chem. Abstr.*, 21 (1927), 2049.

(20) Levin, F. A., *Quart. J. Pharm. Pharmacol.*, 2 (1929), 17.

(21) Kimura, K., and Fujikawa, F., *J. Pharm. Soc. Japan*, 51 (1931), 105.

(22) Wallis, T. E., and Dewar, T., *Quart. J. Pharm. Pharmacol.*, 6 (1933), 347.

(23) Mansfield, J., *Drug. Circ.*, 58 (1914), 240.

(24) Flückiger, F. A., *Schweiz. Wochenschr. Pharm.*, 19 (1873), 600.

(25) Planchon, G., and Collin, E., "Les Drogues Simples," Octave Doin, Editor, Paris, T2 (1896), p. 617.

(26) Greenish, H. G., "The Microscopic Examination of Foods and Drugs," Ed. 3, P. Blakiston's Son & Co., Philadelphia, 1923, p. 117.

(27) Moll and Janssonius, "Botanical Pen Portraits," 1923.

(28) Gilg, Brandt and Schuroff, "Lehrebuch der Pharmakognosie, 1927.

(29) Planchon, L., and Bretin, P., "Précis de matiére médicale," Maloine, Paris, T., 1 (1928).

(30) Judd, D. B., and Kelley, K. L., *Research Paper* RP 1239. Part of *J. Research Natl. Bur. Standards*, 23 (Sept., 1939).

(31) Zufall, C. J., and Burlage, A., THIS JOURNAL 21 (1932), 330.

(32) Clevenger, J. F., *Ibid.*, 17 (1928), 345–349.

(33) "United States Pharmacopœia XI," 1936, p. 473.

(34) Levin, F. A., *Quart. J. Pharm. Pharmacol.*, 2 (1929), 19.

(35) Eames, A. J., and MacDaniels, L. H.,

"Plant Anatomy," ed. 1, McGraw-Hill Book Co., New York, 1925, p. 265.
(36) Benedict, H. M., "Memoirs N. Y. Exper. Agr. Sta.," June, 1915, p. 281.
(37) Ensign, M. R., *Am. J. Botany*, 6 (1919), 3111.
(38) Ensign, M. R., *Ibid.*, 8 (1921), 433.
(39) Zornig and Weiss, *Arch. Pharm. Berl.* 263 (1925), 462.
(40) Wallis, T. E., and Dewar, T., *Quart. J. Pharm. Pharmacol.*, 6 (1933), 352.
(41) Wallis, T. E. and Dewar, T., *Ibid.*, 6 (1933), 349.
(42) Brandes, R., *Arch. Pharm.*, 22 (1827), 229.
(43) Bedford, P. W., "PROC. AM. PHARM. ASSOC.," 1863, p. 211.
(44) Thompson, C. J. S., "Yr. Bk. Pharmacy," 1890, p. 499.
(45) "Schimmel's Report" (Oct. 1911), p. 23.
(46) Gildemeister, E., "Volatile Oils," Ed. 2, J. Wiley & Sons, Inc., New York (1916), p. 639.
(47) Parry, E. J., "Chemistry of Essential Oils," 1918, Vol. I, p. 435.
(48) Bull. Natl. Formulary Comm., 8 (1940), 181.
(49) Kondakow, J., *J. prakt. Chem.*, 54 (1896), 433.
(50) Bialobrzeski, U., *Pharm. Zeit. Russl.*, 35 (1896), 417.
(51) Semmler, F. W., and McKenzie, A., *Berl. Berichte Deutsch. Chem. Gesellschaft*, 39 (1906), 1158.
(52) De Waal, M., *Pharm. Weekblad*, 61 (1924), 185.
(53) Jones, H., *Pharm. J. Trans.*, ser. 3, 9 (1879), 673.
(54) Newcomb, E. L., THIS JOURNAL, 10 (1921), 848.
(55) Squire, P. W., "Companion to British Pharmacopœia," 1916, p. 324.
(56) Sage, W., *Pharm. J.*, 76 (1906), 125.
(57) Holmes, P., *Pharm. J.*, 85 (1910), 69.
(58) Dummer, R. A., *Kew Bull.* (1912), 326.
(59) Mansfield, W., *Proc. N. Y. Ph. A.* (1912), 297.
(60) Jensen, H. R., *Pharm. J.*, 90 (1913), 60.
(61) *Service and Regulatory Announcements, U. S. D. A. F. D.* through *Am. J. Pharm.*, 89 (1917), 549.
(62) Watt, J. W., and Breyer-Brandwijk, "The Medicinal and Poisonous Plants of Southern Africa," E. and S. Livingstone, Edinburgh, 1932, p. 902.

Book Review

Colorimetric Determination of Traces of Metals, by by E. B. SANDELL, University of Minnesota. Interscience Publishers, New York, 1944. xvi + 487 pp., 73 figures, 66 tables. 16 x 23.5 cm. Price, $7.00.

In the past, the analyst in need of a method for a trace element had to go through several laborious steps. First, he had to search the literature for possible methods. He then had to evaluate the practicability of these methods for his particular problem. Finally, unless the element had been well studied previously in the material to be analyzed, he had to develop his own detailed procedure.

Extended search of the literature has been largely eliminated by recently published compilations of colorimetric methods which, however, contributed little to the succeeding steps. Sandell's treatment of colorimetric methods will be found particularly useful to the analyst in the latter steps because he has selected from the large number of methods available those particularly suitable for trace analysis; he discusses the theoretical background so far as known of these methods; and he describes representative applications of each in detail.

The volume is divided into two parts: the first consists of general discussions of methods of separation and analysis of traces, colorimetric principles and colorimetric reagents; the second part consists of analytical procedures presented topically according to elements. All common metals and many of the rarer metals are included. As a typical example, the chapter on copper includes a discussion of methods of separation of this element, extensive discussion of the dithizone and carbamate methods with mention of other methods, and specific procedures for the determination of copper in silicate rocks, steel, water and biological materials. The procedures are in general adaptable to photoelectric colorimeters and spectrophotometers as well as to visual colorimeters or comparators.

The presentation of the theory of at least one method of wide application—dithizone (for Bi, Cd, Cu, Pb, Hg, Ag, Zn)—is unexcelled. The inclusion of a rational system for comparing sensitivities of reagents fills a long-felt want, although the table of sensitivities would perhaps be more useful if presented in terms of molar extinction coefficients rather than as γ required to produce an arbitrary change in extinction. The methods presented for certain elements will no doubt be found inadequate but this is apparently due to lack of intensive research and interest in the element or inherent difficulties presented by its chemical nature.

This volume will find an important place in the analyst's library. Its most important function will be as a handbook rather than as a treatise on colorimetric methods, which is to say that it will have a considerable practical value in the laboratory. And, in common with all significant scientific contributions, it should stimulate further research on important problems.—E. M. SCOTT.